The effect of high gravity on the brewer´s yeast metabolism - physiological studies and "omics" - analyses

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Introduction

Beer is an ancient drink which can be traced back almost 5000 years to Mesopotamia, first mentioned in 2800 BC. Today, beer is one of the beverages with the largest production in the world, with an annual production of 1,767 million hectoliters in 2007, an average annual increase of 2.5 % based on realized production volume and sales for the last five years, and an expected increase at the same rate in the next 10 years. Beer has been an important beverage for thousands of years, first as a way of conserving cereals, later for pleasure.

One of the major players in the brewing process is brewer´s yeast and its importance to brewing was recognized even in ancient times when its biological nature was not known. With the work of Pasteur from 1876 and further pure culturing of brewing yeast cells**,** brewer´s yeast has been recognized, not just as an ingredient, but as an active agent in beer fermentation, being one of the largest contributors to successful fermentation and good quality beer.

Depending on their morphological characteristics, brewer´s yeasts are separated into top fermenting (ale) and bottom fermenting (lager) strains. While ale brewer's yeast strains are closely related to the laboratory strains of *S. cerevisiae*, lager brewer´s yeast strains exhibit more divergent genome. Today, lager beer yeast strains are classified as a part of the *Saccharomyces pastorianus* group and considered a polyploid (in particular allopolyploid) species hybrid of *Saccharomyces cerevisiae* and other closely related *Saccharomyces* species. Nearly 90% of the overall production volumes belong to that of lager beer. Lager brewing yeasts pose unique characteristics that distinguish them from other yeasts and contribute to the uniqueness of the beer fermentation process as a whole. Such unique characteristics are the ability to grow at lower temperature (between 8 °C to 15 °C) and rare sporulation with low rates of viable spores.

Besides using yeast with a complex genetic background, the complexity of the beer fermentation process is further enhanced by the complexity of the media used, the brewing wort. Normally, all-malt wort contains 90% carbohydrates of which the fermentable carbohydrates in order of their concentrations are: maltose, maltotriose, glucose, fructose and sucrose. Up to 25% of the carbohydrates are non-fermentable maltodextrins, and many different process optimisation

strategies have been focused on targeting the maltodextrins and providing more available fermentable sugars for the brewer´s yeast. Moreover, in addition to the complex carbohydrate profile, brewer´s wort also contains complex nitrogen sources. The wide variety of nitrogenous compounds in wort includes amino acids, ammonia, oligopeptides and proteins with longer chains. Brewer´s yeast cannot utilize peptides with more than five amino acid units, therefore, another major goal for fermentation optimisation has been to provide more available assimilable nitrogen for the brewer´s yeast. Both carbohydrates and nitrogen compound uptakes by the brewer´s yeast are ordered processes, starting from the most preferred and moving to the least preferred source. Thus, presence of certain sugars and amino acids leads to inhibition of the uptake of others.

Brewing is both an art and science. Thus, as ancient and well established as it is, the beer fermentation process has high uniqueness and complexity primarily determined by the specificity of the lager brewer´s yeast genome and the composition of the brewer´s wort.

Traditional drivers for technological improvements in the brewing industry have been the need to: improve quality, increase capacity, manage the production costs and compensate the increase of commercial costs in addition to growing governmental and public demand for low environmental impact. One of the first commercial triggers, resulting in remarkable technological improvements in the last 30-40 years was the energy crisis in the 1970´s. As energy and water are the two highest cost factors in brewing operations, the need to produce larger volumes in a shorter time lead to the introduction of the concept of high gravity brewing.

The use of high gravity brewing technology has the advantages of increasing brewery capacity without the need for capital expenditure, reducing the costs of energy and labor (because of the reduced liquid volumes), improving the recovery of ethanol per unit of fermentable sugars and improving the stability of the final beer. A common method to increase the wort concentration without need for modifying the existing brewing capacities is by the use of carbohydrate syrups as liquid adjuncts. However, there is a risk that the use of such syrups will dilute the available nitrogen concentration of the wort and result in a beer with elevated levels of acetate esters and higher alcohols. Other disadvantages are associated with nitrogen limitation, high osmotic pressure at the beginning of the fermentation, high carbon dioxide concentration and high osmotic pressure towards the end of the fermentation and as a resultsluggish and incomplete fermentations. This is an especially critical point considering that in an industrial setting brewer’s yeast is reused several times and high gravity brewing also reduces the cycles in which yeast can be used in subsequent

fermentation rounds. Desired trends for improvement of brewer´s yeast strains used in high gravity brewing are: increased stress resistance, the ability to achieve faster and more complete utilization of fermentable sugars compared to controls, higher viability at the end of the fermentation process, and flavor profiles and flocculation behavior similar to those obtained from brewing at lower gravities.

The development of genome-wide high throughput technologies to identify and map different cellular components and to quantify different types of cellular molecules has offered new possibilities for the study of biological systems. As the lager beer fermentation process is highly complex, both in terms of the complex brewer´s yeast genome and fermentation media (wort), in addition to the limited use of metabolic engineering strategies in industrial beer fermentation, it is of interest to characterise the brewer´s yeast fermentation process and relate the genotype to the phenotype.

With the availability of the *S. cerevisiae* genome sequence together with bioinformatics tools which enable integration and interrogation of large *x-omics* data sets, it is possible to identify high-probability targeted genetic strategies to increase yield, titer, productivity, and/or robustness of existing industrial process. Due to the complex genome nature of the lager brewer´s yeast, associated with its polyploid nature and chromosomal translocations, its sequencing is challenged. This is also the main reason that the *x-omics*[1] characterisation of the brewer´s yeast compared to those of the common *S. cerevisiae* is well behind. Despite the mentioned limitations, characterisation of the transcriptome, proteome and metabolome of the beer fermentation process is highly valuable. Considering that strategies involving genome interrogation (such as metabolic engineering or even in some cases random mutagenesis or) have very limited application on industrial scale beer fermentations due to the current international legislations, the use of "*x*-omics" techniques for characterisation of the brewer´s yeast metabolism is even more appealing. The work presented in this thesis is focused on elucidating the lager brewer´s yeast stress response in high gravity beer fermentation using "-omics" approaches such as transcriptome and metabolome analysis. Model fermentation system simulating as close as possible the large scale industrial lager beer fermentation process have been designed and used to characterise one of the most popular lager beer yeast strains- Weihenstephan 34/70, belonging to the group of *Saccharomyces pastorianus*.

1.1 Overview of the thesis

The work presented in this thesis covers different aspects of the characterization of brewer´s yeast metabolism in the conditions of high gravity brewing with the general objective of gaining novel insights into the brewer´s yeast response to the various stress factors imposed on it. Particular focus is given on evaluation of the effects of nitrogen limitation and glucose repression on the fermentation performance of the brewer´s yeast. In addition, the genome stability of the lager brewer´s yeast is investigated.

Schematic outline of the thesis is given in **Figure 1-1**.

[1] *X-omics* is a general term referring to collection and analysis of any global data set (transcriptome, metabolome, proteome, fluxome, etc.) whereby any type of informational pathway with reference back to the cell´s genome is investigated.

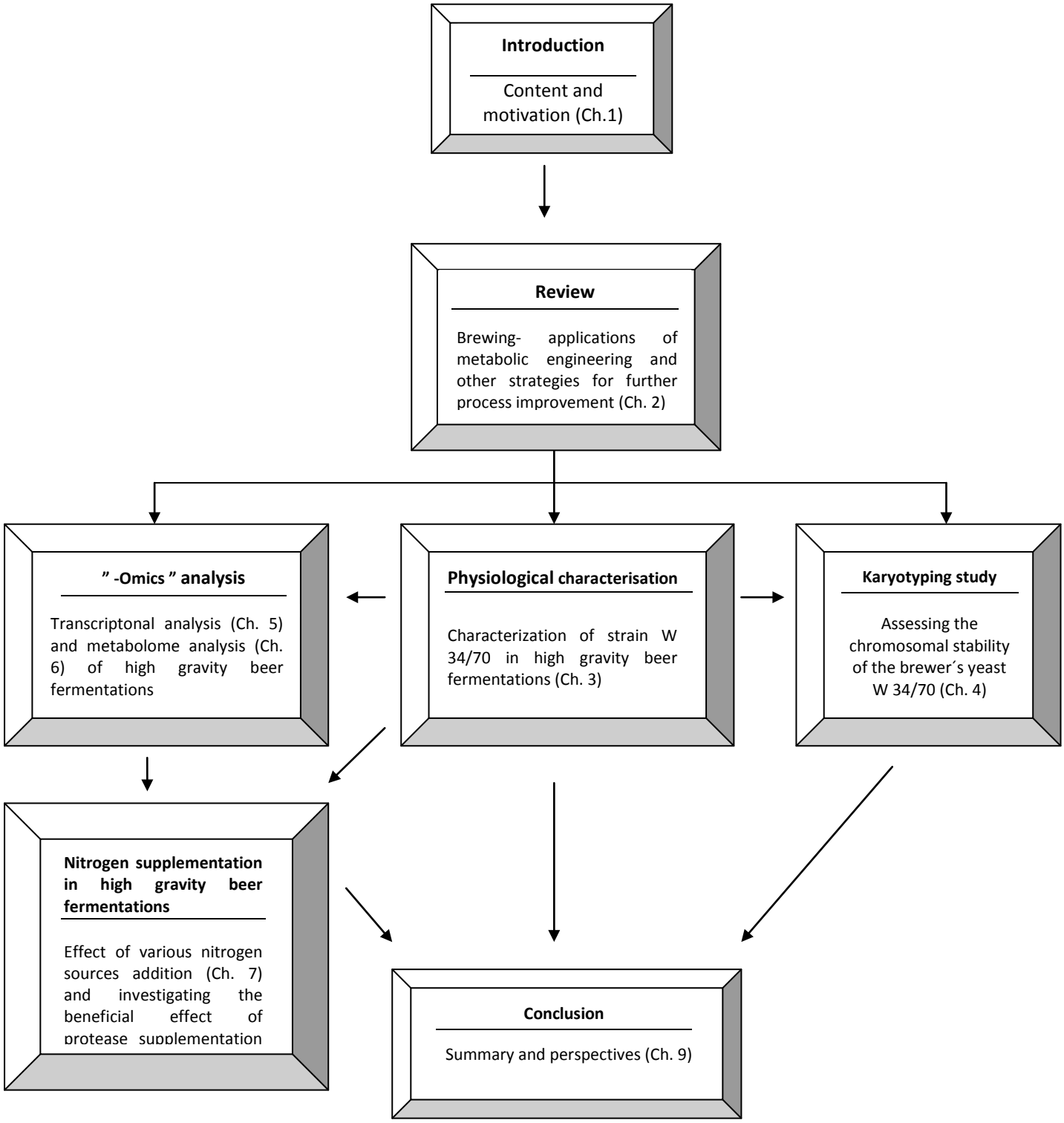

Figure 1-1. Schematic outline of the thesis. The abbreviation "Ch" refers to the chapters in the thesis.

The current chapter (**Chapter 1**) introduces the motivation behind and the flow of the PhD project. **Chapter 2** includes a thorough review f the main up-to-date metabolic engineering process optimization strategies available for the brewing industry. **Chapter 3** presents detailed physiological characterization of the strain Weihenstephan 34/70 at average gravity- 14 °P and at high gravity- 21°P and 24 °P fermentations, achieved with the addition of either glucose or maltose rich sugar syrups (adjuncts). For the fermentations, model fermentation systems on laboratory scale were designed to represents as close as possibly the large scale brewing process. **Chapter 4** approaches the issue of genome stability of the lager beer yeast strains, where karyotypes of strain Weihenstephan 34/70 are followed for several consecutive reinoculation cycles. **Chapter 5** represents a global transcriptional analysis of strain Weihenstephan 34/70 in high gravity beer fermentations. In this study, transcriptome samples from the early exponential phase (phase of exponential cell growth, sugar consumption and product formation) and from the stationary phase (phase of maintenance metabolism, with no cell growth, but still some accumulation of ethanol) of fermentations at 14 °P as well as 21 and 24 °P are characterized and the effects of the growth phase, sugar syrup addition and the interaction of both on the metabolism of brewer´s yeast are discussed. Particular focus is given on the various stress responses and nitrogen metabolism. In **Chapter 6** we discuss the part of the study where the physiological behavior of lager beer yeast strains with different ethanol tolerance was investigated. In addition, metabolome analysis was used to reveal the metabolic response for the well ethanol tolerant strain AJL 3126, the less ethanol tolerant AJL 2252 and the reference Weihenstephan 34/70 from the early exponential and stationary phase at average (14°P) and high gravity (21°P) fermentation conditions. In addition, the metabolome samples for strain Weihenstephan 34/70 were compared to metabolome samples from previously characterized fermentations at 24 °P. The aboved physiological studies in addition to transcriptome and metabolome data revealed the importance of sufficient nitrogen availability and the influence of the wort sugar composition on the uptake of nitrogen compounds by the brewer´s yeast. **Chapter 7** continues this discussion by addressing the effects of the addition of inorganic (ammonium sulphate) and organic (urea) nitrogen sources as well as addition of proteases aiming to increase the release of nitrogen from the wort and their influence on the fermentation performance of the strain Weihenstephan 34/70 at 21 °P high gravity beer fermentations. As supplementation of the wort with the multicomponent protease Flavourzyme showed the most superior fermentation performance in terms of higher specific growth rate and improved wort fermentability, further detailed metabolome and transcriptome analyses from the early exponential and stationary phase of those fermentations

were performed (**Chapter 8**). Finally, **Chapter 9** presents the overall conclusions and comments on future perspectives.

1.2 List of publications

The present PhD project resulted in the following manuscripts and publications included in the thesis:

M. Piddocke, L. Olsson. "Brewing – application of metabolic engineering and other process optimisation strategies for process improvement in beer production", book chapter in "Encyclopedia of Industrial Biotechnology", accepted;

M. Piddocke, S. Kreisz, H. P. Heldt-Hansen, K. F. Nielsen**,** L. Olsson. "Physiological characterization of brewer´s yeast in high gravity beer fermentations with glucose or maltose syrups as adjuncts", in Applied Microbiology and Biotechnology, electronically published 3th of April, 2009, DOI number 10.1007/s00253-009-1930;

M. Piddocke, M. L. Wong, M. L. Nielsen, L. Olsson. Assessing the chromosomal stability of the brewer´s yeast Weihenstephan 34/70, manuscript in preparation.

M. Piddocke, A. Fazio, W. Vongsangnak, M. L. Wong, J. Nielsen, L. Olsson. Transcriptional study of high gravity beer fermentations- the effect of glucose repression and nitrogen limitation, manuscript in preparation.

M. Piddocke, S. Jensen, L. Bonnichsen, R. Festersen, H. P. Heldt-Hansen, L. Olsson. Effect of organic and inorganic nitrogen source additions in high gravity beer fermentations on the brewer´s yeast metabolism, submitted.

M. Piddocke, A. Fazio, W. Vongsangnak, M. L. Wong, H.-P. Heldt-Hansen, C. Workman, J. Nielsen, L. Olsson. Revealing the beneficial effect of protease supplementation to high gravity beer fermentations using "–omics" techniques, manuscript in preparation.

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Brewing-applications of metabolic engineering and other strategies for process improvement in beer production

This chapter is based on the publication:

"Brewing-applications of metabolic engineering and other strategies for process improvement in beer production", in *Encyclopedia of Industrial Biotechnology* (ed. Michael Flickinger), accepted for publication

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Keywords: Brewer´s yeast genetics, classical strain improvement, metabolic engineering, transcriptomics, metabolomics, high gravity, immobilized yeast, enzymes in brewing

Abstract

Today beer is one of the largest beverage products with an annual production world wide of 1 767 million hectoliters in 2007 alone with lager beer production accounting for 90% of the total beer produced. The present drivers for technological improvement in brewing are increased industrial competition and consolidation, constant demand for increased productivity and flexibility, the need for achieving high quality together with low costs and compliance with environmental policies. Using metabolic engineering strategies, improvements of the brewer's yeast fermentation performance has involved increased attenuation rate, improved control of the production of beer flavor metabolites, increased ethanol yields and osmotolerance for high gravity beer fermentations, and reduced ethanol yield for low or zero alcohol beer production. With the availability of the *S. cerevisiae* genome sequence, together with the bioinformatics tools enabling integration and interrogation of large *x-omics* data sets, it is possible to identify high-probability targeted genetic or metabolic strategies to increase yield, titer, productivity, and/or robustness of the existing brewing process. Since the use of GMO strains in brewing is not accepted, mutagenesis remains the alternative approach for obtaining brewer´s yeast variants with superior performances. However, the use of enzymes, often products of genetically modified strains, for brewing process optimization is currently recognized. As brewing is a product based process, introduction of new technologies for process optimization such as high gravity brewing and continuous fermentation using immobilized yeast in brewing lead to improved economics without change in the product characteristics and quality.

Introduction

Beer is an ancient drink which can be traced back almost 5000 years to Mesopotamia. Its first known mention was in the year 2800 BC. Through the centuries, beer, in some form has been served as a common daily beverage in a wide range of cultures and countries. It was provided for the workforces in Mesopotamia and in ancient Egypt, brewed as a household beverage by Germanic and Celtic tribes, and later expanded to a stage of "industrial" production by many Christian monasteries and nunneries where it was produced not only for personal consumption but also for profit. In the 14th century hops were introduced as a key flavoring ingredient (1). Later, Britain, Bohemia and Germany fueled the development of beer brewing techniques. In the 15th century the brewing trade was established as a commercial activity and developed a monopoly on yeast production and also the supply of yeast for baking. Ale brewing and the use of ale yeast strains existed several hundred years before the brewing of lager and the use of lager yeast strains. In most parts of Europe and the world, lager brewing has since almost completely replaced the brewing of ale. During the industrial revolution in the United Kingdom in the 18th and 19th centuries, the development of the commercial brewery was driven by increased urbanization which offered a global market for beer as a widely available consumer product and an opportunity for large scale production (2). By 1830, domestic brewing accounted for 20% of commercial production in the United Kingdom, and by 1900 the annual production of beer by commercial breweries reached nearly 50 million hl.

Certainly, the importance of yeast to brewing and baking was recognized in the ancient times, although its biological nature was unknown. In the first manifestation of the Bavarian beer purity law *Reinheitsgebot* in 1516 which dictated the acceptable ingredients in beer, the exclusive use of hops, malt and water for brewing was prescribed. Yeast was not included as an ingredient as its nature and importance was unknown. With the work of Pasteur from 1876 and further pure culturing of brewing yeast cells, yeast's importance for beer fermentation has been identified and since exploited (3). Brewer´s yeast has been recognized as not just an ingredient but an active agent in beer fermentation, becoming one of the largest contributors to successful fermentation and good quality beer.

In the last thirty years, new yeast strains have been developed to suit the needs of different wort qualities in different parts of the world. Brewing companies have focused on biotechnology to both find new products and more efficient processes to survive in a growingly competitive market. In the 1980s and 1990s the low calorie and low carbohydrate beer trend became increasingly popular. Since then, substantial efforts have been made to develop new yeast strains capable of degrading the unfermentable dextrins into fermentable sugars and to produce a palatable low carbohydrate beer. Further improvement of the performance of brewer's yeast fermentation has involved increased attenuation rates, improved control of the production of beer flavor metabolites such as esters, higher alcohols and sulfur compounds, increased ethanol yields and osmotolerance for high gravity beer fermentations, or respectively reduced ethanol yield for low or zero alcohol beer production.

Today, beer is one of the beverage products with the largest production in the world with an annual production of 1,767 million hectoliters in 2007, an average annual increase of 2.5 % based on realized production volumes and sales for the last five years, and an expected increase in the next 10 years (4). While consumption rates for this period have been flat or declining in Western Europe and North America, the Asian and Eastern European markets continue to grow with especially fast growth in China and Russia.

The present day drivers for technological improvement in the brewing industry are increased industrial competition and consolidation, constant demand for increased productivity and flexibility, and cost control while maintaining quality standards and environmental policy compliance (5).

The purpose of this review is to comment on, and summarize the most advanced applications in the brewer's yeast genetic and metabolic engineering strategies, together with recent process optimization strategies in beer fermentation as well as discuss public perception and governmental regulations on the use of such.

Overview of the brewing process

The brewing process can be separated into four main stages: malting, wort production, fermentation and down stream processing. Schematic overview of the brewing process is present in Figure 2-1. Barley is the main cereal grain used in malting, but in some cases it is mixed with wheat or smaller amounts of sorghum, rye, oats and millets - but only after the malting process is complete. During malting the separate grain types are hydrated (steeped) by immersion in water. After steeping the grains are drained and germinated to a certain extent. During germination the starchy endosperm is partly degraded. After reaching the desired level of germination, the process is stopped by kilning. Kilning is a process of drying in a current of warm to hot air. The resulting malt, in some cases premixed with adjunct, is then milled and suspended in water at controlled rates and temperatures. Adjuncts are materials other than malt that are sources of extract. They are used either to achieve less expensive extract or to enhance certain desirable characteristics of the product (6). Solid or so called "mash tun" adjuncts such as flakes or grits of maize or rice, wheat flour or micronized wheat grains, may be added to the grist. The starch they contain will be degraded by the enzymes of the malt or from supplementary enzymes added to the mash. During mashing, this suspension is heated in a series of stepwise increases in the temperature in order to activate the hydrolytic breakdown of carbohydrate degrading and protein degrading malt enzymes. At the end of mashing the sweet wort is separated from the un-dissolved solids called spent grains and further boiled with hops. Other soluble adjuncts such as sugars or syrups are dissolved in the wort during boiling. In addition to those sugars, caramels or other compounds can be added to adjust color. The wort contains four main fermentable sugars- maltose, maltotriose, glucose and fructose, assimilable nitrogen in the form of amino acids and oligopeptides and proteins, minerals and vitamins, together with some minor growth factors. Prior to fermentation, the wort is aerated, followed by inoculation with yeast. Oxygen is required in the initial stages of the fermentation and proper aeration is essential for sufficient cell growth and successful fermentation (7). The fermentation is divided into primary and

secondary fermentations. Primary fermentation may continue for several days depending on the type of beer being produced during which the yeast utilizes the available carbohydrates in the wort and ethanol and carbon dioxide are produced. During secondary fermentation (also called maturation), the flavour and aroma profile of the “green” beer is refined. This process takes 1-3 weeks and it is performed at lower temperatures, allowing the remaining yeast cells to reduce the off-flavours and produce the desired flavour compounds (8, 9).

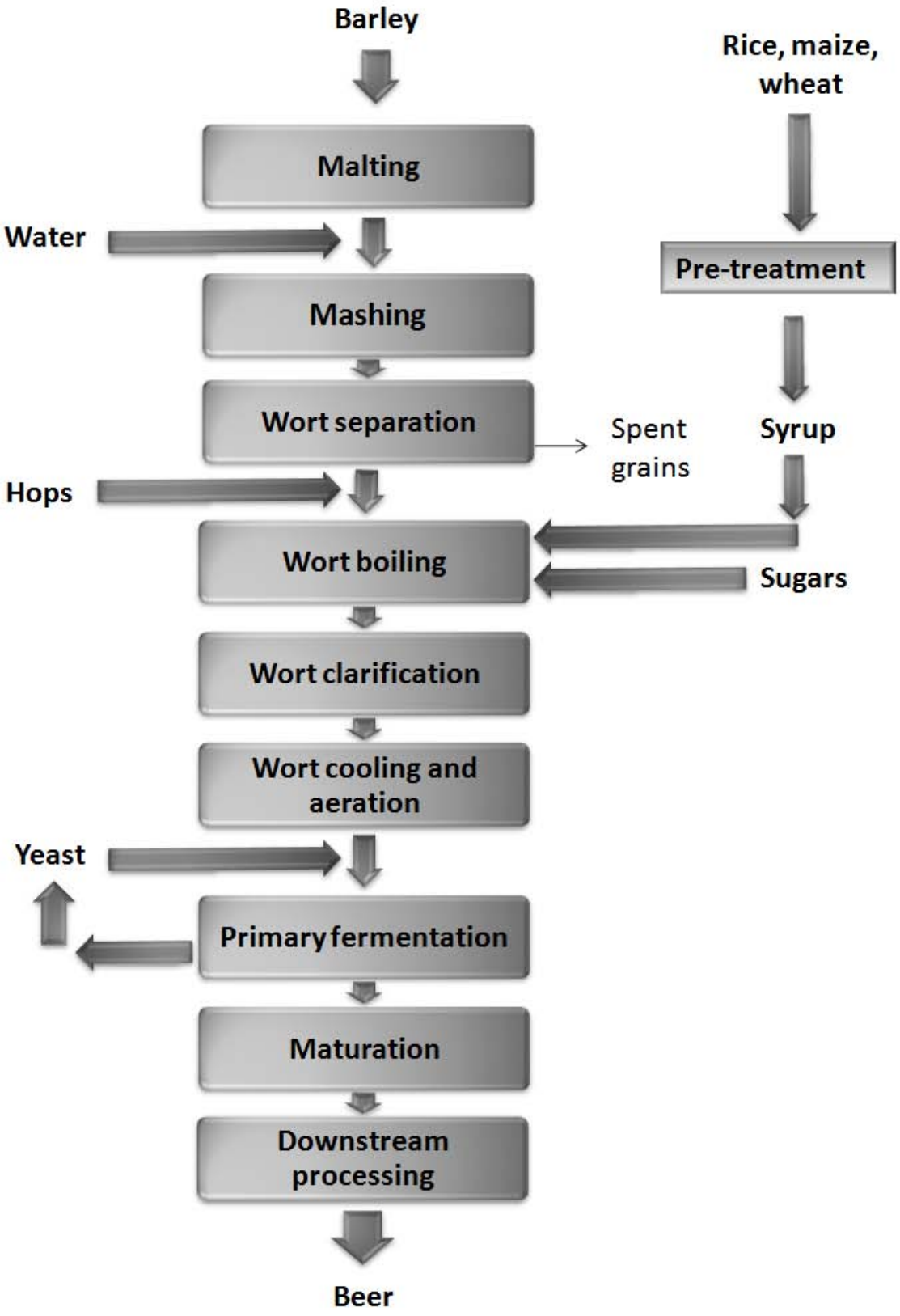

Figure 2-1. Schematic overview of the brewing fermentation process.

Brewer´s yeast genome

Saccharomyces cerevisiae has been the dominant organism used for millennia in food fermentations. Its main advantages are associated with its ability to effectively transform sugars into ethanol and carbon dioxide, and into a large number of secondary flavor compounds, but at the same time be able to resist stress caused primary by temperature, osmotic pressure and ethanol toxicity (7).

In 1883, Emil C. Hansen from the Carlsberg laboratory discovered a method for using single cell cultures of yeast in beer production. This led to a revolution in brewing because, for the first time, pure culture yeast brewing became possible. The first pure cultured lager brewing yeast was named "Bottom Fermenting Strain #1" and later renamed to *Saccharomyces carlsbergensis*. Currently, most of the lager brewing yeast strains are thought to be close relatives to this strain (8).

Depending on their morphological characteristics, brewer´s yeasts are separated into top fermenting and bottom fermenting yeasts. Top- fermenting yeasts are diverse groups of polyploid strains closely related to the laboratory strains of *S. cerevisiae* (10, 11). They are used for the production of "ale" type beer at temperatures of 20° to 25°C. Ale brewing predates lager brewing and thus tends to display greater genome variability (12). The bottom fermenting lager yeast strains were initially characterized as *S. carlsbergensis*, and later renamed as *S. pastorianus.* They are used for the production of lager beer with fermentation at a lower temperatures - between 8° to 15°C and normally do not produce offspring. While the production of ale has a shorter maturation time, or no maturation at all, lager beer production requires a maturation time of up to a few weeks (13).

Currently, lager brewing accounts for more then 90% of the world´s beer production and therefore most of the research has been focused on lager yeast (8, 13). Lager beer yeast strains pose unique characteristics that distinguish them from other yeasts. As reviewed by Hansen and Kielland Brandt, 2003 (8 and references there in) a number of studies examining essential genes have shown that in almost all cases two divergent types of the genes of interest are present in the genome, of which one closely resembles the *S. cerevisiae* gene and the other shows divergent patterns.

Through the years the taxonomy of the lager beer strains has undergone several changes. Initially, they were included as a part of the taxon *Saccharomyces cerevisiae*, but later they were classified as a part of the *Saccharomyces pastorianus* group and considered a polyploid species hybrid of *Saccharomyces cerevisiae* and other closely related *Saccharomyces* species (8).

Based on kar-mediated single chromosome transfer, many of the lager yeast chromosomes such as chromosomes III, V, VII, X, XII and XIII have been studied. Based on the single chromosome studies, the chromosomes derived from lager beer yeast strains have been found to be of three types: homologous (*S. cerevisiae* type), homoeologous (non *S. cerevisiae* type), and mosaic types, composed both from homologous and homoeologous segments (8 and references there in).

Determination of the ploidity of the lager brewing yeast, the types of chromosomes and the number of copies they exist in is of essential importance for establishing optimal strategies for its targeted

molecular breeding (13) and further metabolic engineering. A number of studies to determine the mosaic structure of the lager beer yeast chromosomes, using Southern Blot hybridization, restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) (14; 15) have been done. Using HIS4 and LEU2 probes with RFLP, lager and ale strains could be differentiated, while the use of Ty1 probes enables in most cases differentiation between closely related lager species (15). During most studies of specific genes of interest from the brewers yeast genome two hybridization and restriction patterns are found- one very similar to *S. cerevisiae*, usually named *S. cerevisiae*- like type (Sc-) and the other showing divergent patterns named - *S. pastorianus* (Sp-), lager (Lg-) or *S. carlsbergensis* (CA-) like type (8; 13).

The identity of the non-*S. cerevisiae* type is not fully revealed. Among the suggested candidates were strain CBS 1503 from *S. monacensis* (16) and *S. bayanus* strain CBS 380 (17-19). While for the first one it has been proved that it also contains Sc-type genomic DNA (20- 22) for the later one two versions for many of the genes - one identical to the non-*S. cerevisiae* type of genes and another about 7% divergent have been reported (19- 22). Most recently two isolates of *S. bayanus*- IFO 539 and IFO 1948 have been reported to present pure genetic lines, carrying the content from only the non-Sc genome (8).

The nucleotide sequences for several of the lager brewing yeast genes have been determined and compared to the equivalent nucleotide sequences from *S. cerevisiae*. In all cases high homology was observed in the coding regions for the two types, while the non-coding sequences showed less similarity. A BLASTn based homology search analysis has demonstrated that the lager strains' specific genes exhibit 85-98% homology to those of *S. cerevisiae* (12). For example, in the study of the amino acid uptake and flavor formation, two types of the *BAP2* genes which encode branch chain amino acid permeases were found (23). One of the *BAP2* genes**,** Lg-*BAP2* is identical to that of *Saccharomyces bayanus* by-*BAP2*-1 (by-denoting *S. bayanus* type). The other *BAP2* gene of the lager brewing yeast cer-*BAP2* is identical to that of *S. cerevisiae*. The amino acid homology between the two genes was 88%. Transcription has shown that the gene expression of the two genes is differentially regulated. Studies on the essential brewer´s yeast metabolism genes- *ILV2, ATF1, MET2, MET10, ACB1, HO, MET14* and *MXR1* also proved 78-88% similarity on nucleotide levels and 76-97% on amino acids levels between the two types of genome (8, 13). The high or complete identity of *S. cerevisiae*- like type of genes to the respective native genes of *S. cerevisiae* have been confirmed with partial or full sequencing for several of the *S. cerevisiae*- like type genes.

Another important consideration is the copy number of each type of a particular gene present in the lager brewer's yeast genome. From a production strain of *S. carlsbergensis*, deletions of the two wild type alleles were constructed in vivo in order to study the consequences of eliminating the *ILV2* gene function. Results from southern blot hybridization proved that the strain contained two copies for each of the two versions of the *ILV2* gene. A Number of studies have proved the general allotetraploid structure of the lager brewer's yeast, its irregularity in the chromosome setup in some cases, and possible aneuploidity for some chromosomes or chromosome regions (8, 13, 24).

It is also suggested that unlike the haploid or diploid strains, tetraploids require homologous recombination for survival and this potentially reflects the increase in the spontaneous genetic instability in lager brewer's yeast. At the same time, although lacking mating mechanisms, the genomic rearrangements in lager strains clearly exhibit adaptive evolution and this may be further enhanced by the allopolyploid nature of the genome (12).

With advances in DNA sequencing technologies in recent years, the possibility of whole genome sequencing of the first lager beer yeast strain, the popular Weihenstephan 34/70, became more feasible. First reported by Nakao *et* all. in 2003, a combination of shotgun sequencing and cosmid libraries sequencing was used to achieve 348 001 total sequence reads of the genome of the lager beer strain Weihenstephan 34/70, resulting in 160 million base pairs of DNA, corresponding to the 6.5-fold coverage of the genome (25). The minimum total size of the lager brewer's yeast found was almost double to the size of the *S. cerevisiae* genome and corresponded to 23.2 million base pairs. Mapping contigs of the lager brewing yeast genome to those of *S. cerevisiae* also revealed three kinds of chromosomes- Sc-type, non-Sc- and various mosaic types. In the mosaic type some of the chromosomal breakpoints appeared inside the ORFs, therefore proving the existence of hybrid ORFs. In most of the cases, because of low nucleotide similarity to *S. cerevisiae* ORFs, these ORFs were classified as non-Sc. More recombination break points were expected close to the telomere regions as these are subject to frequent rearrangements (13, 26). To fully reveal the exact number of copies for each chromosome, comparative genomic hybridization has been suggested, using DNA microarrays containing all of the lager brewing ORFs. The signal intensity of each probe relative to each ORFs will reflect the copy number of each ORF.

Microarray based comparative genomic hybridization (CCGH) has been used to study the relative copy number of the individual *S. cerevisiae*-like genes in two lager beer yeast strains 6701 and CMBS-33 compared to a haploid laboratory *S. cerevisiae* strain S-150. It was found that the copy number for large contiguous groups of *S. cerevisiae* genes was similar for both lager yeast strains and the majority of loci where the copy number changes were conserved in both strains. However, the increased copy number of the *S. cerevisiae*-like genes to the right of the *MAT* locus did not result in any significant increase in the gene expression of the studied time-points –day 1 and day 8 of the fermentation. This observation leads to the hypothesis that some form of dosage compensation might take effect in the lager strains (27).

Further on, the chromosome integrity of both the stress tolerant strains and their parents in single rounds of fermentation and under a variety of environmental stresses has been examined. Especially in the case of high gravity and for higher than normal temperature fermentations, the lager yeast genome exhibited enormous plasticity and genetic changes as a response to the environmental stress (28).

Study of the mitochondrial DNA from lager yeast revealed a size of 70 kb - similar to the reported mitochondrial DNA from *S. bayanus* (66.6 kb) and somewhat smaller compared to that of *S. cerevisiae* (85.5 kb). The gene order and the sequence identities of the mitochondrial genes in the

lager brewing yeast were very similar to that of *S. bayanus*. These findings lead to the hypothesis that lager brewing yeast inherited its mitochondrion from the non-*S. cerevisiae* ancestor (12).

Strain development by classical methods and metabolic engineering

The targets for the improvement of brewer's yeast strain can mainly be qualified in two categories- producer driven and consumer driven trends for improvement (summarized in Figure 2-2). The producer driven improvement trend involves improvement of the fermentation performance and simplification of the process such as the improvement of dextrin and maltotriose utilization and increased ethanol production. The consumer driven trend for improvement is mostly focused on improvement of product quality and hygienic characteristics - such as the reduction of off-flavors, flavor formation, modified or enhancement of desired flavor formation, or resistance to contamination by wild yeasts (11, 29). However the two trends are closely interconnected and some of the desired improvements fall in both categories (Table 2-1).

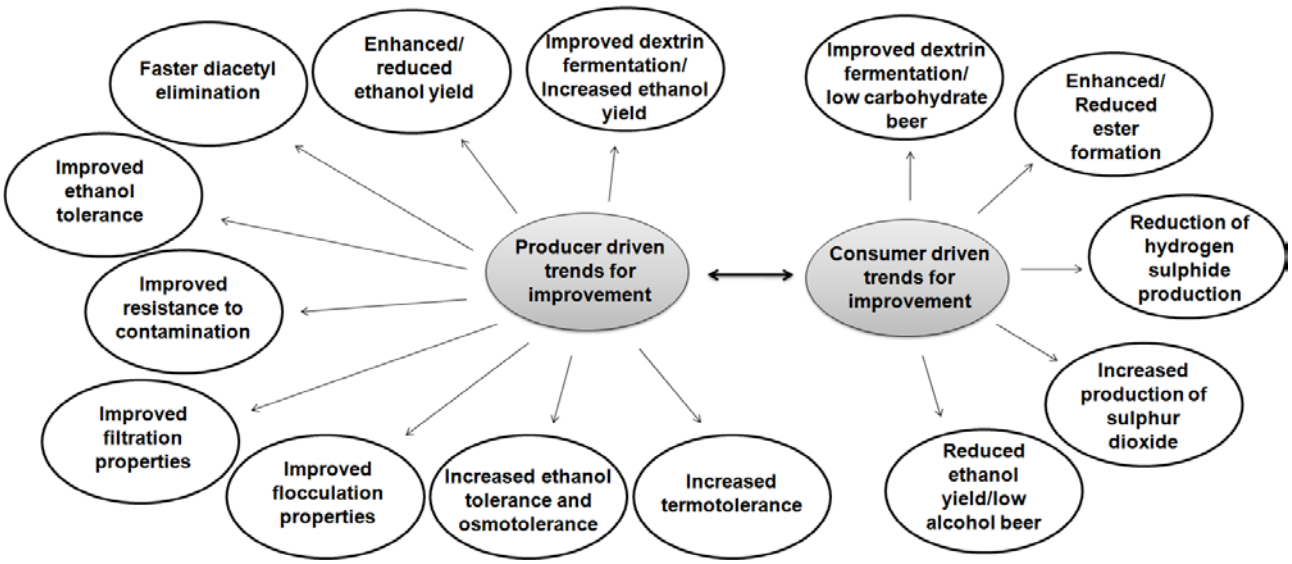

Figure 2-2. Some of the most important trends for improvement in beer fermentation.

Table 2-1. Examples of producer driven and consumer driven trends for improvement.

Producer driven trends for improvement ⟷	Consumer driven trends for improvement
Improved dextrin fermentation/ Increased ethanol yield	Improved dextrin fermentation /Low carbohydrate beer
Enhanced/Reduced ester formation	Enhanced/ Reduced ester formation
Faster diacetyl elimination	Reduction of hydrogen sulphide production
Improved ethanol tolerance	Increased production of sulphur dioxide
Reduces risk of contamination by wild yeast	Reduced ethanol yield/ Low alcohol beer
Improved filtration properties	
Improved flocculation properties	
High gravity beer fermentations/Increased ethanol and osmotolerance	
High temperature fermentation/ Increased thermotolerance	

Classical methods

The ability of numerous industrial strains of *Saccharomyces cerevisiae* to transform sugars efficiently into ethanol, carbon dioxide and numerous secondary flavor compounds and its ability to withstand stress caused primarily by temperature, osmotic pressure, ethanol toxicity and competitive bacteria and wild yeasts, has been determinative for the dominance of *S. cerevisiae* in baking and alcoholic beverage production. The classical methods for strain improvement use two

main principles: the exchange of DNA between individuals by hybridization, protoplast fusion, cytoduction or mutations (spontaneous or targeted) and subsequent further selection for desired traits (11). For successful classical selection it is also necessary that the new genotype results in phenotype with enhanced desired characteristics and that the individual mutant can be separated from the rest of the population (30). Desired characteristics can be introduced by mating the brewer´s yeast strain with other, non brewer's yeast strains. Such hybrids are valuable for obtaining targeted breeding, where yeast with certain properties is needed, as most often only one copy of each type for a given gene is present. Other methods used for breeding of lager yeasts are "rare mating" of well characterized haploid yeasts with brewer´s yeast and further protoplast fusion (8). One example is the development of a brewer´s yeast strain carrying both antibacterial properties and the 'killer factor', which was obtained by mating a respiratory deficient strain with antibacterial properties and a killer strain and further fusion of the resulting hybrid with brewer´s yeast (31). Another example is the construction of amylolytic brewing yeast which focused on rare mating or protoplast fusion of diastatic wild yeasts with brewing strains and further backcrossing. This approach also led to the introduction of undesired trends such as phenolic off-flavour formation associated with the wild yeast. The main disadvantage of classical methods is the possibility of unpredictable gene transfer with little to no understanding of the specific genetic perturbations (8).

Mutagenesis

Mutagenesis can be achieved by the use of UV-irradiation or chemical agents. Both types of mutagenesis are followed by screening and selection procedures. Mutagenesis has advantages such as not requiring prior information for the selected genes and strains, the genomic mutations are induced relatively randomly and at high frequencies and that the product of mutagenesis is not considered genetically modified and special labeling is not required. Mutagenesis could be challenging when proper selection procedure needs to be designed for selection of only several cells carrying the desired phenotypes among a vast number of cells (30).

UV mutagenesis has been used in a number of studies to find resulting strains with favorable fermentation characteristics and better stress tolerance. An example is the selection of better-performing variants under very-high-gravity conditions using the commercial lager brewing strain CMBS33 as a starting strain. By using UV irradiation with different strengths on the cells harvested from the stationary phase of the beer fermentations, a series of mutants were created. The control strain and the pool of mutated cells were re-innoculated several times in very high gravity wort > 22ºP. Two of the variants showed faster fermentation rates and more complete fermentation with higher final attenuation under those conditions. Their improved performance was further verified in high gravity fermentation trials (32).

A non-metabolizable analogue of 2-deoxyglucose has been used to isolate *S. cerevisiae* strains defective in glucose repression. The selected mutants showed decreased glucose repression, resulting in reduced fermentation times during high gravity fermentation trials. Based on the knowledge that acetyl-CoA synthethase gene (*ACS1*) is a subject to glucose repression and

assuming that constitutive expression of *ACS1* plays a role in the decrease in acetic acid production, a mutant with low acetic acid and high volumetric ethanol productivities has been obtained and its superior performance has been verified in high gravity beer fermentations (33).

Despite their limitations, mutagenesis and other classical methods have proven to be commercially successful and their use has been applied for obtaining a number of brewer´s yeast strains with enhanced fermentation characteristics. Even more importantly mutagenesis is still the main tool for obtaining strains with desired characteristics for many industrial food biotechnology processes. Yet, with the need for further, more targeted strategies, the era of metabolic engineering has emerged and also found application in the development of brewer´s yeast strains.

Metabolic Engineering of Brewer´s yeast

As an alternative to the classical genetic methods, metabolic engineering strategies available today enable us to perform directed genetic perturbations to improve the production strains of interest. With the *S. cerevisiae* genome sequence publicly available since 1996, such modification has become feasible. Metabolic engineering involves identification of specific genetic modifications (gene deletions, over-expression or modulation) and implementation of these modifications using molecular biology tools. Such genetic perturbations lead to redirection of fluxes and enhanced production or robustness of a given product or organism (34). Most of the metabolic engineering strategies in brewing have been focused on the major player in the process- brewer´s yeast. Despite the complex genome nature of brewer´s yeast, there have been a number of successful metabolic engineering strategies focused on increasing ethanol yield, improved sugar utilization in the fermentation process, and reduction of off-flavor formation, improving the flocculation and filtration properties. Overview of the brewer´s yeast metabolism with focus of the most important metabolites in beer fermentation is present in Figure 2-3.

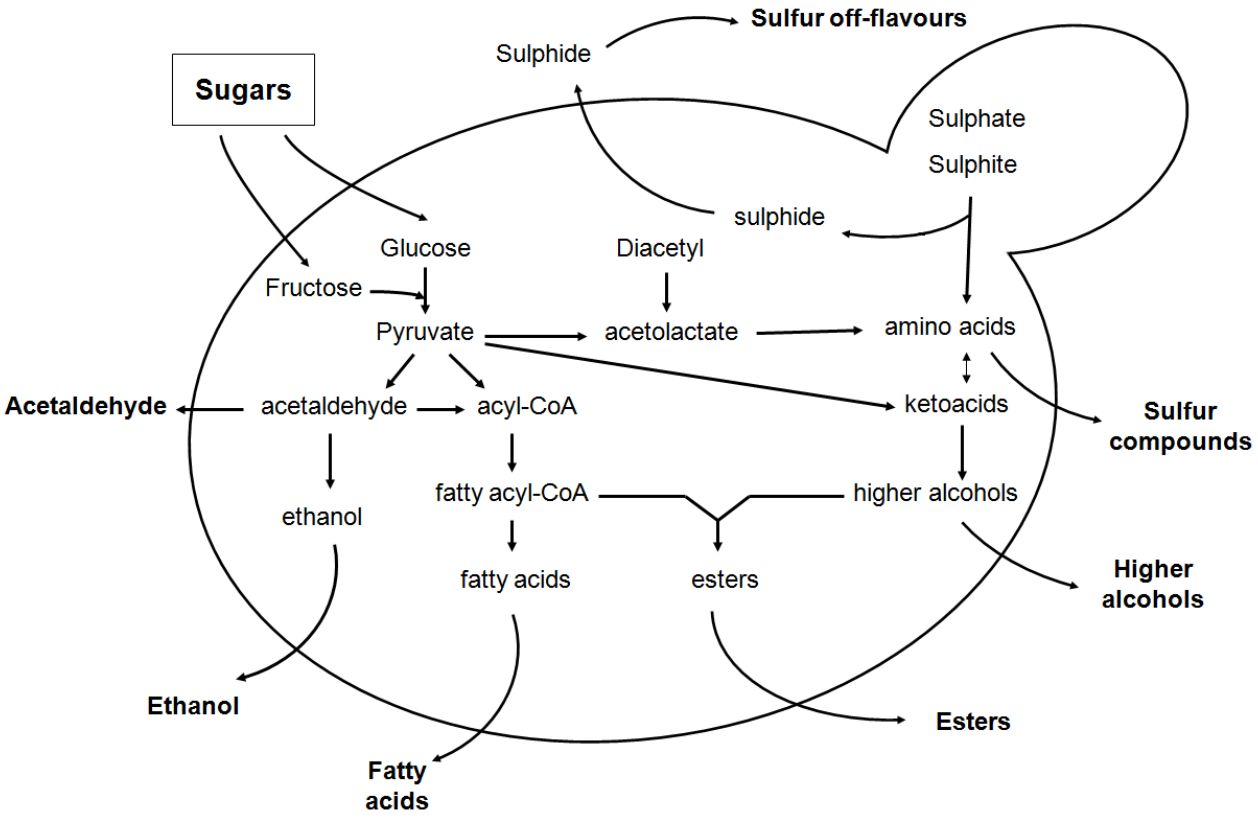

Figure 2-3. Overview of the brewer´s yeast metabolism.

As brewer´s yeast is most often tetraploid in its genome nature, to target specific genes in the lager brewing yeast, it is necessary to isolate, characterize and sequence both the Sc- and the non Sc-versions of the gene and in order to eliminate certain gene functions, most often two copies from each of the *S. cerevisiae* and the non- *S. cerevisiae* type have to be disrupted. As selective markers normally used in haploid *S. cerevisiae* strains cannot be applied in lager yeast, the alternative is to use at least four dominant drug resistant markers, in the case of the brewer´s yeast tetraploid strain (13). Some of the most popular metabolic engineering strategies for improved brewer´s yeast characteristics are present in Table 2-2.

Table 2-2. Popular metabolic engineering strategies for improved of brewer´s yeast characteristics.

Strain	Modification	Improvement	Reference
BH-84, *Saccharomyces cerevisiae*, Lager brewer´s yeast	Overexpression of *MALT* gene	Enchanced maltose fermentation	**45**
NCYC 1324, Lager brewer´s yeast	High copy number plasmid containing a *DEX* gene	Improved dextrin fermentation	**113**
RSA1, Brewer´s yeast strain	Plasmid integration of glucoamylase gene from *Bacillus amyloliquefaciens*	improved dextrin fermentation	**39**
Sake yeast *Saccharomyces cerevisiae*	Overexpression of *LEU4* from multiple copies in the genome	Increased isoamyl alcohol production	**61**
S. pastorianus BH-225	Overexpression of the leucine transporter *BAP2*	Increased isoamyl acetate production	**23**
KBU 001 *S. ovarum*	Overexpression of *ATF1*	Increased isoamyl acetate production	**114**
Brewer´s yeast YSF5	Disruption of *ILV2*	Lower α- acetolactate decarbohylase activity	**50**
S. uvarum S1	Overexpression of *ILV5* from a plasmid	Higher reductoisomerase activity, Lower diacetyl levels	**48**
S. carlsbergensis	Deletion of MXR1, encoding the reduction of DMSO to DMS	50-80% lower DMS content	**59**
S. cerevisiae 9B *S. cerevisiae* NA5-2C *S. cerevisiae* NA21-2C	Overexpression of *NHS5* and *STR4*, encoding cystathionine β-synthase	Reduction of hydrogen sulphide production	**55**
BH-84, lager brewer´s yeast	Increased copy number of MET25	Reduction of hydrogen sulphide production	**54**
S. carlsbergensis	Partial or full deletion of *MET10*	Reduction of hydrogen sulphide production and accumulation of sulfite, increased flavor stability	**52**
S. uvarum S1	Overexpression of *ILV5* from a plasmid	Higher reductoisomerase activity, Lower diacetyl levels	**115**
Saccharomyces carlsbergensis # 385	Overexpression of *ILV5* into the genome	Up to 60 % reduction in diacetyl production	**49**
Lager brewer´s yeast strains VTT-A-63015 VTT-A-66024	Expression of the *Enterobacter aerogenes* or *Klebsiella terrigena α-ALDC* gene from a multicopy plasmid	Almost complete reduction in diacetyl production	**116**
S. carlsbergensis IFO 0751	Expression of the *Enterobacter aerogenes-α-ALDC* gene in the genome	Reduction in diacetyl production	**117**
Lager brewer´s yeast strain K1084T	Expression of the *α-ALDC* gene from *Acetobacter aceti* ssp. *xylinum* in the genome	>70% reduction in diacetyl production	**118**
S. carlsbergensis Sa-07256	Overexpression of *GPD1*	Six fold increased glycerol yield, 18% reduced ethanol yield	**51**
Haploid, non flocculent *S. cerevisiae* FY23 strain	Expression of *FLO1* under *HSP30* promoter active in the stationary phase	Controlled flocculation towards the end of the fermentation	**68**
	Expression of and fungal *Trichoderma reesei* β-glucanase	Improved wort´s viscosity and filtration	**119**

Desired fermentation trends- sugar utilization

One of the early targets for yeast metabolic engineering has been the development of amylolytic yeast strains with the ability to hydrolyze residual dextrins in wort. As dextrins are non fermentable carbohydrates and they account for around 25% of the sugars present in the wort, many metabolic engineering strategies have been focused on the expression of various glucosoamylases genes. Strains with improved ability to utilize maltodextrins will result in beer with lower calorie content. *Saccharomyces diastaticus*, a variety of *Saccharomyces cerevisae*, is a glucoamylase producing yeast and because of its close relationship to brewer's yeast, it has been an obvious source of the glucoamylase gene in several strategies (35). Similar to the synthesis of maltase, the synthesis of glucoamylase in yeast is controlled by a set of at least three polymeric genes- *STA1*, *STA2* and *STA3*. Improved dextrin fermentation has been achieved by using low or high copy plasmid expression of various glucoamylase genes. Initially, classical genetic approaches have been used to transfer the *STA*1 gene which encodes amylolitic properties from *S. cerevisae* var. *distaticus* to brewer´s yeast, however its transformation lead to co-transformation of the *POF1* gene responsible for phenolic off-flavor. To overcome these problems, the *STA2* gene from *S. distaticus*, which encodes amyloglucosidase was cloned on a multicopy plasmid and was used to transform a commercial lager yeast strain (8, 11). The resulting strain had improved fermentation performance and better capability to utilize maltodextrins, while the flavor and aroma profile of the final beer remained similar. An amylolytic brewing yeast *Sacharomyces pastorianus*, was constructed by disrupting the α-acetolactate synthase gene and introducing the α-amylase gene as a selective marker. The resulting recombinant strain was able to utilize starch as the sole carbon source and its α-acetolactate synthase activity was lowered by 30% (36). In a different study the α-acetohydroxyacid synthase (AHAS) gene *ILV2* was disrupted and the dextranase gene (LSD1) from *Lipomyces starkeyi* was introduced as a selective marker. Subsequent fermentation trials with the tranformants strains confirmed dextranase activity and lower AHAS activity and, for some of the transformants, the residual sugar content was reduced by 20-25% compared to the host strain (37).

Multiple integration of *S. cerevisiae* var. *diastaticus STA1* into the genomic rDNA genes resulted in improved dextrin fermentation and increased the ethanol production by 10% (8, 38). Alternative sources for producing various glucoamylases are also several of the *Aspergillus* species. Another improvement made to the brewer´s yeast ability to ferment maltodextrins was with the integration of genes from *Aspergillus niger* and *Aspergillus awamori* (11), which encode the hydrolysis of α-1,6 linkages of dextrins into the genomes of lager yeast. Insertion of the glucoamylase gene from *Aspergillus niger* in all three copies of the brewer's yeast HO gene resulted in a 50% decrease in dextrin content and a 20% increase in ethanol production. The drawback of using the *A. niger* glucoamylase was its high thermostability and therefore the inability to deactivate it by pasteurization. Even further, genes with both α-1,4 and α-1,6 activity from the *Schwanniomyces occidentalis* have been expressed in brewer's yeast. In this case the expressed enzyme also had the advantage of being thermostable, but could be inactivated by pasteurization at the end of the process (8).The most efficient brewers yeast strains to utilize maltodextrins have been achieved by coexpression of both the *STA2* gene from *S. distaticus* coding for a glucoamylase and the *AMY* gene from *Bacillus amyloliquefaciens* (39).

Besides utilization of dextrins, other desired trends for improved sugar utilization in brewing are associated with improved utilization of maltose and maltotriose and reduced glucose repression. Maltose is the main sugar present in wort, accounting for around 60% of the fermentable sugar, followed by maltotriose (15-20%) and glucose (10-15%). The sugar consumption in beer fermentation is an ordered process. In the course of beer fermentation maltose and maltotriose utilization do not commence until approximately half of the glucose present in the wort is depleted (40). The main reasons for this phenomenon are glucose repression of the maltose and maltotriose transporters and of the α-glucosidases (maltases) responsible for maltose and maltotriose uptake, and glucose mediated inactivation of the transporter protein. This is of particular importance for high gravity brewing, where very high glucose concentrations are present in the wort. While many *S. cerevisiae* strains consume maltose well after the exhaustion of glucose, maltotriose is a less preferred sugar and some yeast strains are not able to utilize it at all (41). Complete maltose and maltotriose utilization is an important aspect of the brewing process with respect to beer quality and production cost. High residual maltoriose concentrations at the end of fermentation results in lower ethanol yield and an atypical flavor profile of the final beer. Prior to the development of metabolic engineering strategies for improved sugar utilization, major research was focused on genetic engineering studies and biochemical analysis of the mechanisms and complexity of maltose transport and utilization. While glucose enters the cells through the facilitated hexose transport system, the utilization of maltose and maltotriose requires active transport of those sugars through the plasma membrane and further hydrolysis by cytoplasmic glucosidases (maltases). Both maltose and maltoriose transport have shown complex kinetics involving both high and low affinity transporters. All characterized α-glucoside transport systems so far are H^+ symporters using the electrochemical proton gradient to transport these sugars inside the cell. However, maltose and maltotriose are transported by different permeases, but further intracellularly hydrolyzed by maltases. The presence of at least one of the five highly homologous and unlinked *MAL* loci – *MAL1-MAL4* and/or *MAL6* is required for maltose utilization (42). Each locus contains at least one copy of the set of three different genes which encode a maltose permease (*MALx1*, with x standing for one to five loci), maltase (*MALx2*) and a positive regulatory protein (*MALx3*) - inducing the transcription of the permease and maltase in the presence of maltose. Yet, some other loci are only partially functional and may contain some, but not all of these genes, particularly the *MAL1* and the constitutive *MAL4* loci. Other loci can contain additional copies and/or alleles of some genes, such as a constitutive regulator found in some of the *MAL6* loci (41). Since it has been determined that *MAL1* strains are unable to consume maltotriose from the wort, the knowledge about the molecular identity of the maltotriose transporters advanced when the permease gene named *AGT1* was characterized in a partially functional *mal1g* locus (43). The Agt1 transporters have a relatively broad specificity including maltose, maltotriose, sucrose, trehalose, melezitose, and α-methylglucoside, with trehalose as the preferred substrate. It has also been reported (41) that to efficiently consume and ferment maltose and maltotriose from a synthetic or rich medium, *AGT1* permease must be expressed constitutively by the yeast cells. As brewer´s yeasts are aneuploid and lager yeast strains are alloploid, quantification of the *MALx1*gene doses in both ale and lager strains has been attempted (44). During growth on maltose the lager strains expressed *AGT1* at low levels and *MALx1* genes at high levels while the opposite was observed for the ale strains. It was found

that maltose transport in the ale strains was mainly mediated by broad specificity *AGT1*-encoded transporters, while the transport for the lager strains used predominantly *MALx1* encoded transporters with high specificity for maltose. To improve maltose assimilation efficiency, constitutive expression of the *MAL* genes with high copy number plasmids in the brewing yeast strain of *S. cerevisiae* has also been attempted (45). Over-expression of the maltose transporter gene with a gene promoter not repressed by glucose improved the maltose consumption rate in high gravity beer fermentations significantly. Each of the *MAL* genes was expressed together with a constitutive promoter of the yeast glyceraldehyde 3-phosphate dehydrogenase gene and introduced into the yeast.

Flavor profile

Although some of the flavor compounds are directly derived from the raw materials used for wort production, brewer´s yeast fermentation has the most significant impact on flavor development. To a large extent, the overall balance of flavor metabolites is largely a consequence of the combination of yeast strain and wort composition. The yeast metabolites contributing to the beer flavor belong to diverse group of metabolites including esters, organic acids, aromatic alcohols, medium chain aliphatic alcohols, carbonyls and various sulphur containing compounds. More then 200 flavour components can be identified in beer (46) and while some contribute positively to the flavor profile of the final beer, others are off-flavors giving a negative impact even when present in concentrations of nanograms.

With increasing demand for process optimization and enhanced productivity, studies of the flavor compounds and the factors that influence their formation and removal have been increasingly important. Metabolic engineering strategies have also been used as tools to obtain brewer´s yeast strains which enhance desired flavors or reduce off-flavor formation. One of the main reasons for the requirement for a long lagering period at the end of the primary beer fermentation is the non-enzymatic, slow conversion of α-acetolactate to diacetyl, which is further enzymatically converted to acetoin and subsequently to 2,3-butandiol. Diacetyl is involved in the amino acid metabolism and its threshold value is much lower than that of acetoin. From a metabolic engineering point of view one way to avoid the off-flavor formation caused by diacetyl is by the introduction of a heterologous α-acetolactate decarboxylase which enables the transformed strains to have an alternative route of degradation of α-acetolactate by producing acetoin directly from α-acetolactate. For example, the acetolactate decarbohylase gene (ALDC) from *Enterobacter aerogenes* has been integrated into the genome of brewer´s strains under the control of the *ADH1* promoter. In subsequent fermentation trials, the resulting strain produced less diacetyl compared to the parent strain (8, 47). Potential application of such a strain in the brewing industry would result in reduced maturation time from weeks to hours. Another successful strategy to bypass the diacetyl formation was to increase the flux through the valine biosynthetic pathway. By over expressing the *ILV5* gene (48), or by integration of multiple *ILV5* copies into the genome (49), a two fold reduction in diacetyl formation can be achieved.

A brewer's yeast strain, free of vector sequences and drug-resistant genes has been constructed by disrupting the α-acetohydroxy acid synthase (AHAS) gene (*ILV2*) and introducing the *Lipomyces starkeyi* dextranase (DEX) gene (*LSD1*) as a selective marker. The resulting recombinant strain could grow on dextran as the sole carbon source and it showed lower AHAS activity. The resulting recombinant strains showed improved fermentation performance and reduced residual sugar content by 20-25%, compared to the host (50). Random mutagenesis in combination with over-expression of specific gene encoding enzymes involved in the biosynthesis of valine has also been used for the selection of strains with reduced diacetyl formation. Partial inactivation of the *ILV2* gene function has been carried out by selection of spontaneous dominant mutants which are resistant to sulfometuron methyl. The selected isolates exhibited slow growth on isoleucine- and valine-deficient medium and resulted in strains with low acetolactate synthase *ILV2* activities and reduced carbon flux towards α-acetolactate formation (48).

In a different study the effects on assimilation of branched chain amino acids and production of the respective higher alcohols was investigated (23). The constitutive expression of the *BAP2* gene which encodes branched chain amino acid permease in brewer´s yeast resulted in accelerated rates of assimilation for leucine, valine and isoleucine. The production of isoamyl alcohol derived from leucine was increased, but changes in the production of isobutyl alcohol derived from valine or active amyl alcohol from isoleucine was not observed.

In the high gravity brewing approach, higher alcohol concentration is the desired trend. There is also a low-alcohol beer market which requires a product with a low final ethanol concentration. The conventional methods of reducing ethanol concentration at the post fermentation stage or by manipulation of the fermentation process are expensive and result in beer with modified and undesirable flavor profiles. Using the metabolic engineering approach, ethanol production could be reduced by redirecting the carbon flux towards other fermentation products than ethanol. A shift of carbon flux towards glycerol has been achieved by over-expression of glycerol-3-phosphate dehydrogenase, an enzyme catalyzing the reduction of dihydroxyacetone phosphate (51). The resulting strain reduced ethanol content by 18% and modified the profile of the final beer. Moderate increase in the acetate, succinate and 2,3- butandiol, as well as significant increase in the acetaldehyde, acetoin and diacetyl levels were observed.

Reduction of hydrogen sulphide production

Sulphite acts as an antioxidant and a stabilizing agent for flavor. It is formed by the yeast during assimilation of inorganic sulphate and it is an intermediate in the synthesis of sulphur containing amino acids, but its concentrations normally present in the cells are low (35). Furthermore, sulphite is converted to sulphide by sulphite reductase and then into homocysteine by homocysteine synthetase.

To accelerate the sulphite concentration and respectively to improve the flavor stability, a strategy of eliminating the sulphite reductase activity was undertaken. Sulphite reductase is a tetramer with a

two α- and two β- subunits, encoded by the *MET10* and *MET5* genes, respectively. Because of difficulties in disruption of four functional copies of the *MET10* gene in the allotetraploid strain, instead allodiploid strains, obtained as meiotic derivatives of the brewing yeast strain were used. The allodiploids contained two homeologous alleles of the *MET10* gene, one similar to the one found in *S. cerevisiae* and the other specific to *S. carlsbergensis*. By crossing the resulting diploids containing non-functional copies of the genes, new allotetraploid strains with reduced or abolished *MET10* function were generated. The *MET10* gene is part of the sulphite reductase enzyme and partial or full disruption of this gene showed substantial increase in the sulphite accumulation and abolished sulphide production (52).

Hydrogen sulphide is an important off-flavor producing compound to identify as it can negatively influence the beer's flavor profile. Typical concentration of this compound in beer is in the range of 1-20 µg/l, with a threshold value of 5 µg/l. In beer the hydrogen sulphide is present partly in a bound form, but its total level may exceed the threshold level. Lager yeasts are less efficient in reducing sulphur dioxide to hydrogen sulphite than ale yeast. During vigorous fermentation hydrogen sulphide will, to a large extent, be removed with the carbon dioxide (53). It is produced by sulphite reduction and is condensed to activated homoserine inside the cells. It is believed that methanethiol and ethanethiol are derived from it (8). *MET25* is a gene which encodes homocysteine synthetase. By overexpressing *MET25*, a ten fold reduction of the amount of hydrogen sulphide present in the media was observed (54). *STR4* is a gene which encodes cystathionine β-synthase and converts homocysteine to cystathionine. It has also been reported that overexpression of the *STR4* gene resulted in suppressing the formation of hydrogen sulphide (55).

Similar strategies for enhanced sulphite production have relied on the inactivation of *MET2*, the gene which encodes *O*-acetyl transferase. This enzyme catalyses the synthesis of *O*-acetyl homoserine, which in turns binds to hydrogen sulphide to form homoserine. Inactivation of *MET2* impedes cysteine formation and derepresses the genes for sulphite biosynthesis, but also leads to accumulation of hydrogen sulphide (52).

Increased production of sulphur dioxide

One of the most studied off-flavor compounds related to aging is *trans*-2-nonenal, an aldehyde with a threshold taste value in the range of nanograms/liter. This compound is important for the stale, cardboard flavor in the final beer when present at levels as low as 0.035 µg/L and it is formed by oxidation of linoleic acid (ref, Lermusieau et al., 1999). It is generally agreed that *trans*-2-nonenal potential is generated during wort production, initiating the *trans*-2-nonenal formation at the later stages of beer storage. It has previously been demonstrated that around 70% of the *trans*-2-nonenal released during beer staling was initially produced during boiling and the remaining 30% was initially produced during mashing (56). In wort, *trans*-2-nonenal forms Schiff base structures (imines) with amino acids or proteins. The resulting adducts are partially lost in the brew house (during the beer fermentation), but the residual adducts protect *trans*-2-nonenal from the reducing activity of yeast and further enhanced by the low pH, free *trans*-2-nonenal will be released by acidic

hydrolysis in the beer during storage (56). Furthermore, part of *trans*-2-nonenal is reduced to nonenol by the yeast during fermentation and it is reoxidized to *trans*-2-nonenal during beer storage (57).

In order to avoid the formation of off-flavors related to aging, the beer needs to be kept continuously cool and exposure to stirring, shaking and light should be avoided as well. Since the cooling process, especially for extended periods, is costly and difficult to maintain, an alternative way to reduce aldehyde formation is by the formation of flavor inactive compounds with sulphite. In beer fermentation, sulphite has the role of an antioxidant and, by forming non-volatile trans-2-nonenal adducts together with the aldehydes, it also has the role of a flavor stabilizing agent (58). In an attempt to increase the sulphur dioxide production, the *MET3* and *MET14* genes which encode ATP sulphurylase and APS kinase have been cloned and overexpressed in brewing yeasts from a multi-copy plasmid. An alternative approach involved disruption of either the *MET2* or *MET10* genes. In the case of *MET10* disruption (52), both decrease in the synthesis of methionine and consecutive derepression of the sulphate pathway and increase in the sulphur dioxide concentration was observed. While in the case of *MET2* disruptants, both sulphur dioxide and the level of hydrogen sulphide increased, the later as a result of the normal function of the sulphite reductase coded by the *MET10* gene.

Dimethyl sulfide reduction

Dimethyl sulfide (DMS) is another important sulfur compound found in beer. In the range between its threshold level of 30 µg/l to 100 µg/l, DMS contributes to the distinctive taste of some lager beers whereas above 100 µg/l, DMS may impart a “cooked sweatcorn” flavor which is usually unwanted. One source of it is derived through thermal degradation of S-methyl-methionine during wort preparation, but a substantial part of it in the finished product comes from the brewer´s yeast’s metabolism by the NADPH dependent conversion of dimethyl sulfoxide (DMSO) to DMS. Methionine sulfoxide (MetSO) reductase isolated from yeast was suggested to be identical to dimethyl sulfoxide reductase, as it has a higher affinity for MetSO that DMSO and MetSO inhibit the DMSO reduction. The effect of the methionine sulfoxide enzyme activity on the DMS production was investigated (59). In *S. cerevisiae*, *MXRI* encodes methionine sulphoxide reductase activity. Its respective analog in lager yeast *S. carlsbergensis* is *MXRI-CA*. The gene was sequenced and showed 88% identity to the *S. cerevisiae MXRI*.

The allodiploid deletion mutants of *S.carlsbergensis* have been constructed from inactive deletion alleles for both genes. Further cross of such mutants resulted in allotetraploid *S. carlsbergensis* without or with only a partially present, *MHRI* gene. DMS was not formed if there was no active *MXRI* present, but some DMS was produced if at least one copy of the active *MHR-CA* was left. While (fermentation trials with) the reference strain resulted in DMS content of the final beer of 54.2 µg/l, the resulting mutants produced beer with DMS content in the range of 28.1-35.1 µg/l, but without significant changes in the concentrations of other flavor compounds.

Enhanced production of flavour compounds

Alcohol acetyl transferases (AATS) are well known and studied enzymes involved in the ester synthesis. They catalyse the formation of acetate esters from two alcohol molecules and acetyl-CoA. Alcohol acetyl transferases in brewer's yeast are divided into three main groups: AATase I, Lg-AAT I- closely related homologue to AATase I, and AATase II. The three groups are encoded by *ATF1*, *Lg-ATF1*- the close homologue to *ATF1*, and *ATF2*, respectively. *ATF1* and *ATF2* are present both in ale and lager strains, while *Lg-ATF1* is found only in lager brewing strains. Homology based search of the *S. cerevisiae* genome has not revealed other genes with homology to *ATF1* or *ATF2*. Furthermore, a possible alcohol acyl transferase, Eht1p (ethanol hexanoyl transferase), involved in the formation of medium chain fatty acid esters, has been described. To investigate the role of the three alcohol acetyl transferases in volatile ester production, the respective genes have been individually over-expressed or deleted in both laboratory strains of *S. cerevisiae* and in a commercial brewing strain (60). GC-MS analysis of the fermentation products showed that besides strongly influencing the production of ethyl acetate and isoamyl acetate, the atf1p and atf2p are also responsible for the formation of a number of less volatile and higher molecular weight compounds such as propyl acetate, isobutyl acetate, pentyl acetate, octyl acetate and phenyl ethyl acetate. The double deletion strain *atf1*Δ*atf2*Δ did not produce any isoamyl acetate, but still produced some amount of the other esters such as ethyl acetate, propyl and isobutyl acetate. The results from the double deletion mutant proved that atf1p and atf2p are responsible for the total cellular isoamyl alcohol acetyl transferase activity, but yet there are unknown ester synthases responsible for the formation of the other esters. Additionally, the over-expression of different alleles of *ATF1* and *ATF2* led to different ester profiles indicating that they might be mutations in the ATF genes (60). One approach of enhanced ester formation is through increased flux towards the precursors for the ester production- the respective amino acids and higher alcohols. To increase the isoamyl acetate levels, in a different strategy, extra copies of the *LEU4* gene in the *S. cerevisiae* genome were introduced (61). The over-expression of the *LEU4* gene lead to increased isoamyl alcohol and increased isoamyl acetate production. As this method also interferes with the metabolism of amino acid and higher alcohols, an alternative approach was the over-expression of one or more ester synthesis genes. Over-expression of the *ATF1* gene resulted in strong increase in the production of ethyl acetate, isoamyl acetate and 2-phenylethyl acetate (30, 62).

Resistance to contamination

For brewing, as well as for other alcoholic beverage fermentations, there is a possibility of contamination with wild yeast and dominance of wild yeast over the inoculated pure culture yeast strain. As a defense mechanism, many wild, contaminating strains produce zymocin, a toxin which targets and kills sensitive strains. In an effort to improve the yeast resistance towards spoilage organisms, several studies focused on the expression of heterologous genes which encode antimicrobial enzyme activities in the genome of *S. cerevisiae*. Some examples applied in wine yeast strains were the expression of a lysozyme encoding gene from chicken eggs (63) in the

genome of *S. cerevisiae* or zymocin genes from the *Pichia* or *Hanseniapora* species, bacteriocin genes from *Pedicoccus acidilactici* and *Leuconostoc carnosum*.

In brewer's yeast strains, by expressing the genes coding for zymocin production and immunity, increased resistance to contamination has been obtained (11, 64).

Filtration

Some brewing practices, or the use of some malt qualities or grain batches, may result in wort, and subsequently in beer, with high viscosity and filtration difficulties. During grain germination, β-glucan, a major component of the endosperm wall, can be degraded by β-glucanase enzymes. Insufficient β-glucanase acitivity during malting results in an excess of β-glucan in the wort. The barley gene which encodes β-glucanase has been characterized and expressed in a *S. cerevisiae* strain (65). As barley β-glucanase had lower thermal stability compared to the bacterial or fungal ones, this strategy was further optimized to increase its thermal resistance (66). Commonly, bacterial (*Bacillus subtilis*) or fungal (*Thricoderma reesei*) amylases are used commercially as an additive to the mash to overcome this problem, but cheaper solutions can be offered if the yeast strains are made capable of secreting β-glucanase during the course of the beer fermentation. Strategies including both expression of genes which encode bacterial (*B. subtillis*) β-glucanase from a multi copy plasmid and a copy of the fungal (*T. reesei*) *EG1* gene integrated into the chromosome have been reported. The later method resulted in almost complete digestion of β-glucanase and significant reduction in beer viscosity (11).

Flocculation

Yeast flocculation is an asexual, reversible, and calcium dependent process in which yeast cells adhere to form flocculi consisting of thousands of cells (67). It is an important process in the brewing industry as it provides an easy, effective and low-cost way to separate the yeast cells from the beer. While most of the lager beer yeast strains are naturally flocculent (bottom fermenting), ale strains are non flocculent (top fermenting). While flocculation is not a desired trend at the beginning of the fermentation, towards the end of the fermentation yeast should ideally exhibit strong flocculation. The yeast cells should not flocculate before the fermentation is completed, since premature flocculation might lead to incomplete or slow fermentation, which in turn results in beer with low alcohol content, unsatisfactory aromatic characteristics, and the possibility of off-flavors (68). As many of the industrially used brewing yeast strains do not have optimal flocculation properties, metabolic engineering strategies leading to improved flocculation are desirable. In the 1950´s two independent studies reported the existence of flocculation genes determined by genetic crosses. Depending on the recognition mechanism for the cells to flocculate, the division of yeast strains in two groups derived from two different lectin binding mechanisms has been suggested (67). One group was determined as the Flo1 phenotype containing the *FLO1* gene and all other

genes that are known to be involved in flocculation. The group showed a manno-specific lectin mechanism. The second group was named the NewFlo phenotype involving the majority of brewery ale strains with unknown genotypes, showing a manno- and gluco- specific lectin mechanism. The two phenotypes were distinguished by sugar, salt and low pH inhibitions, protease sensitivity and selective expression of flocculation. Currently, at least 35 known genes are involved in flocculation. In summary, the genes responsible for flocculation are four dominant genes- *FLO1, FLO5, FLO9* and *FLO10*, the *FLO2* gene, the transcriptional regulator *FLO8*, the semi-dominant *flo3*, the recessive *flo6* and *flo7*. Mutations in several other genes are found to be responsible for flocculation. Furthermore, a third flocculation phenotype has been suggested in which flocculation is not sensitive to mannose and is also independent of Ca^{2+}. This factor could be dependent on the expression of the single, dominant *FLO1* gene (69).

The best known among the flocculation genes is *FLO1*, a dominant gene situated at the right arm of chromosome 1. As expression of *FLO1* results in flocculation, it is believed that the controlled expression of this gene will also result in flocculation. By integration of the *FLO1* genes under the regulation of the ADH1 promoter, a flocculating phenotype has been obtained from non-flocculating yeast (70, 71). However, constitutive expression of *FLO1* is not a desired trend for brewer´s yeast, as it is not suitable for most of the real brewing applications. In a further metabolic engineering strategy, the wild type *FLO1* promoter of non flocculating haploid yeast strain was replaced by a construct consisted of *SMRI-410* marker gene and the *HSP30* promoter (64). This gene is induced by factors which occur late or towards the end of the fermentation. The flocculation rate of the resulting transformants was comparable to that of the flocculent wild type strains. The slower fermentation rate of the transformants and lower concentration of free cells in the second half of the fermentation could be compensated with higher pitching rates (68).

Genetics and metabolic engineering strategies for improved barley characteristics

The desired characteristics for malting barley are high extract yields, free from contaminating microorganisms, and reasonable enzyme activity for α-amylase, β-amylase, proteases and β-glucanases. Conventional breeding has been relatively successful in producing cultivars meeting the requirements for good barley culture, but the use of modern metabolic engineering approaches offers new opportunities for obtaining barley with enhanced characteristics (72).

The transformation of barley is challenged by the fact that cell and tissue cultures are needed for successful transformation and it is strongly dependent on the barley variety. In 1990 a particle bombardment technique was used for the transformation of the first transgenic barley plant (72 and references therein). Later on, other techniques used for transformation were electroporation and polyethylene glycol mediated gene transfer, transformation with thermotolerant bacterial gene, fungal genes, or an engineered native barley promoter. Still, one of the most promising techniques achieved has been *Agrobacterium* transformation, resulting in transformation frequency as high as 4% of the treated barley embryos (73). The transgenic numbers in the different lines ranged from a single copy insertion to at least ten copies. The genetic improvement of enzymes essential to the

brewing process has been an important goal in barley biotechnology. For improvement of β-amylase thermostability in barley seeds, random mutagenesis and site-directed mutagenesis have been used to achieve substitution of seven amino acids of the original barley β-amylase. The resulting mutant β-amylase gene has improved thermostability by 11.6°C compared to the original enzyme and it has been successfully integrated into the barley protoplast under the control of the barley´s β-amylase promoter. Study of four consecutive generations showed that the transgene was stably transmitted and thermostable β-amylase was synthesized in the off-spring (74). As metabolic engineering has an advantage with its targeted approach and introduces a specific gene of interest into the barley´s genome, most research has been focused on introduction of thermostable bacterial β-glucanase into the genome. But introduction of fungal, or engineered native barley genes has also been reported. The integration of the fungal gene inserted in the barley successfully produced heat stable β -glucanase, resulting in reduced wort viscosity (72).

Genetics and metabolic engineering strategies for improved hops characteristics

In brewing, the female influorescences of the hop plant *Humulus lupulus* L. are used to add flavour and bitterness to the beer (75). Hops contain two types of bittering acids: α- and β- acids. The group of α-acids give the beverage distinct flavor as well as promote the yeast brewing process and hinder bacterial growth. β-acids have a greater effect on inhibiting microbial growth, but influence the bitterness to a much lesser extent. Different α to β ratios affect the physical characteristics of hops and their use. The traditional hybridization approach for developing of new hops varieties is a long process which can take up to 10 years. Typical stages involve the selection of promising parents, growing seedlings, selection based on disease resistance, followed by cone production and chemical analysis. In the next steps selected plants are propagated by root splitting or meristem culture. Further selection is made on the basis of yield, disease resistance and chemical analysis. Further field trials are then done with pilot plan brewing and commercial brewing trials. Although some research has been focused on obtaining hop strains with enhanced α- and β- acids production, currently, GMO strains for hops production are not used industrially. Instead molecular markers are used in agricultural genomics, linking inheritable traits such as resistance, sex, and quality to allow early evaluation of a promising breeding material without laborious screening procedures. Molecular markers are fragments of DNA that may vary in size among the individuals and are used as an experimental probe to trace an individual. Each marker is a specific genetic trait. Molecular markers are used to identify DNA associated with desired characteristics such as high α- and β- acids content, storage stability, high yields (as, for example, producing plants with short internodes), disease resistance, low co-humulone, high xanthohumol and fine aroma (76). Marker assisted selection is particularly useful for hops due to their high levels of heterozygosity. It allows breeding lines from crosses of powdery mildew resistant wild hops and susceptible varieties to be examined. In the cross combination of two resistance genes, phenotypically resistant seedlings carrying both resistance genes could only be identified by marker analysis.

GMO and public perception

In 1993, Brewing Research International, UK obtained the first recombinant Brewer´s yeast strain and received official approval for commercial use in 1994. However, this strain has not been used commercially. The strain contained an amylase gene from *Saccharomyces diastaticus* together with a gene for copper resistance. Fearing negative consumer reaction, the industry did not adopt the genetically modified strain in the brewing industry. The same was valid for the bread and wine industry. After commercial application of GM plants took place, public resistance towards GM food became even more evident. A representative survey by Eurobarometer in 2001, covering a sample of 16,000 Europeans with questions on scientific developments and technology, showed generally positive attitude towards the technology. However, a reluctance for the use of GM applications in food remained. Especially in the case of beer production, the benefits of GMO produced beer were considered to be marginal, abstract or only on the producers' side (77). In general, despite the major advances in biochemistry, molecular biology, fermentation technology and biochemical engineering, the adoption of new techniques within the brewing community has been slow, mostly hindered by the unwillingness to change existing proven techniques. Trust in centuries old proven and successful techniques makes for a deserved stubbornness towards innovation. One of the main concerns about the use of GMO is the close relation between some of the food microorganisms and their pathogenic relatives, for example, *Candida albicans* and *Aspergillus fumigatus*. If genetically modified microorganism contain drug resistance genes, there is a possibility for gene transfer from the GM organism to the pathogenic bacteria and fungi. Overcoming this problem will require the funding of alternative approaches for gene modification and the elimination of the use of drug resistance markers in GMO for food applications. For example in Japan, the GM guidelines provided by the government effective April 2001, requires safety assessment of genetically engineered food microorganisms, but exempts the "self –cloning" techniques in which genes of the microorganism are cloned within the microorganism itself. If the microorganism does not contain "foreign" DNA, there is no chance of drug-resistance gene transfer to pathogenic microorganisms and no fear of toxic protein or allergen production caused by a recombinant strain (78). In the long term, an efficient way to promote a rational attitude in the general public towards gene technology is education on different levels with an emphasis on the functionality of the genetic changes. Fundamentally, a lack of understanding and knowledge of the basic biological principles is the main reason for the negative public perception. The public perception could also be positively influenced in the process of marketing of such a product by clearly emphasising the beneficial consumer characteristics as for example- improved flavour characteristics, reduced calories or environmental impact (13). In a step towards positive public attitude of the use of GMO strains it will be also beneficial if further research efforts are focused on evaluating the risk and studying the consequences of the use of GMO strains in the respective food product. Clear distinction between the presence or absence in the final product of recombinant material and the characteristics of such material should also be made available to the broad public.

The new era- the use of enzymes in the brewing process and their governmental regulation

In the food industry, enzymes can be used as alternatives for traditional chemical-based technology and can substitute the use of synthetic chemicals in many different process applications. Their advantages are associated with more specific modes of action, reduced formation of waste byproducts and as a result, improved environmental performance of the production process such as lower energy consumption and biodegradability. According to the Impact assessment concerning the proposal for a European parliament en Council Regulation on food enzymes from 2006, the worldwide market on food enzymes is dominated by several multinational companies of which 60% to 80% belongs to 4 companies (79). This distribution is as followed: 35-50% belongs to Novozymes A/S, 15-20% to DSM Food Specialties and 10-20% to Genencor-Danisco A/S. According to the same report the world market value of food enzymes for 2006 is estimated to be 650-750 million Euros per year. According to the Market Research Report on "Enzymes for Industrial Applications", published by Business Communications Company, Inc., from December 2004, (79) an annual growth of $50 million US is expected in the global food enzyme market for the period 2004 to 2009.

Some of the needs and directions for development are reflected in the leading enzyme producing companies' strategies:

Novozymes A/S, 2008: *In an effort to keep low prices despite increases in raw material prices and to keep constant prices for the end consumer while maintaining rising profits, our company offers new solutions by utilizing enzymes in the brewing industry. The main targets for the currently available enzymes in brewing are focused on higher extract yields, improved attenuation control, longer beer filter cycle runs, shorter cooking cycles, and reduced beer losses and maturation time* (80).

Genencor Danisco A/S, 2008: *Added early in processing, our enzymes are no longer detectable in the final beer or alcohol. Only their effects remain, ensuring optimum stability and shelf life. We have developed a specialized range of enzymes to maximize brewery efficiency and create stable beer with great flavor and clarity. Consumer demand for greater choice and quality has sent brewers worldwide on a never-ending quest to create specialty beers and develop new styles with exclusive flavor characteristics. The addition of our heat-stable enzymes ensures efficient starch extraction, wort viscosity reduction and a sufficient level of free amino nitrogen - all in support of fermentation* (81).

Based on EU legislation, the biotechnologically produced food enzymes obtained from GMOs (82) follow three main categories:

- Food processing aids- most enzymes fall under this category and are used in the food industry as food processing aids. They are still not regulated at EU levels.
- Food additives- the use of enzymes as food additives is less common in practice, but fully regulated by EU legislations through the positive list system (83).

- General chemical legislation- as enzymes are also chemical substances, in some cases the enzymes produced from genetically modified microorganisms might also fall under the EC legislation aimed at regulating chemicals.

According to the directive 90/219/EEC, any activity in which microorganisms are genetically modified and "cultured, stored, transported, destroyed, disposed of or used in any other way", and for which specific containment measures are used to limit their contact with and to provide high levels of safety for the general population and for the environment, follows the scope of the GMO food and feed regulations. Based on the GMO´s directive, notification of the first time use of such an organism should be done by reporting such use to the local national authorities. Exemptions from such notification exist in cases when the GMOs are obtained through the use of induced mutations, cell fusion including protoplast fusion or by self cloning.

Before a new enzyme introduced into the market, it has to be authorized based on a demonstration that there is a technological need for it and the consumer must not be mislead. Such products must undergo safety assessments including technical reports with results obtained from research and development releases in order to evaluate the products impact on human health and the environment (84). Once approved, the authorized enzyme is listed on The Positive List (Community List). The Positive List includes the name of the enzyme, its origin and purity, the conditions of use, and any restrictions of sale. When it comes to labeling the enzyme in the final food product, the enzyme does not need to be named if it will be used as a processing aid. For enzymes not used as processing aids, labeling similar to food additives such as named category and specific name or E-number is required. According to the GMO legislation 1829/2003 of the EU, the standing committee agreed that food produced by fermentation using a genetically modified microorganism which is kept under contained conditions and it is not present in the final product is not in the scope (85). Most of the enzymes used in brewing belong to the classes of α-amylases, β-glucanases, pullulanases, cellulases and proteases. They are often a product of a genetically modified strain from fungal (*Aspergillus niger*), bacterial (*Clostridium thermohydrosulfuricum*) or plant (*Carica papaya*) origin. The function of α-amylases and pullulanases is to increase the yield of fermentable sugars by breaking the bonds from the non-reducing end of the long maltodextrin molecules and release more available maltose and free glucose molecules into the wort. Such beer yields in higher final alcohol content and reduced final carbohydrate content. β-glucanases are used to improve the filtration and viscosity of the wort and α-acetolactate decarbohylases is used to reduce the amount of diacetyl from the final beer. Improved beer stabilization is achieved by proline specific endo-protease by removing the polyphenol-protein complexes, thus preventing the formation of protein chill haze in finished beer.

As in the EU, enzymes used in the brewing industry in the rest of the world should be approved by the local authorities and listed on an equivalent to The Positive List. While in France, Japan and China special approval is needed if the enzyme is produced by GMOs, for the rest of the EU countries as well as the USA, Canada, Brasil and Australia there is no such requirement. According to regulations in the USA, the use of generally recognized as safe enzymes does not require prior approval by the FDA or special labeling. However, every substance added to food

needs to have GRAS status or to be an approved food additive. Evaluation and approval before marketing is required only in the cases when the introduced gene encodes a product that previously have not been a component of any food product. For the countries of the European Union, Brasil, Japan and Australia, final beer should be labeled as a GMO product if GM raw materials have been used (83).

Brewing fermentation optimization

In the today's competitive market there is a continuous demand for increased productivity and high quality beer at low cost. As brewing is generally product based and not process based, introduction of new technologies for process optimization should lead to improved economics without change in the product characteristics and quality. Here we will focus on two of the most common process optimizations achieved in brewing- high gravity brewing and continuous beer fermentations with the use of immobilized yeast.

High gravity brewing

Traditional brewing uses wort with a specific gravity of 12°-14°Plato and it is fermented to produce beer with roughly 5% ethanol content (vol/vol). 1° Plato corresponds to 1 gram of extract per 100 grams of liquid solution, where extract include both fermentable sugars and non fermentable carbon sources such as dextrins and β-glucan (32). The relatively high ethanol concentrations formed during fermentation promotes increased precipitation of polyphenol protein material, thus high gravity produced beer has better colloidal stability then standard gravity fermented beer (86). The higher ethanol yield also improves the microbiological stability of the beer. High gravity brewing requires the use of wort with higher density and because of the higher ethanol concentrations produced, at the end, the beer is diluted to the desired ethanol content. The use of high gravity brewing technology has the advantages of increasing brewery capacity without the need for capital expenditure, reducing the cost of energy and labor (because of the reduced liquid volumes), improving the recovery of ethanol per unit of fermentable sugars and improving the stability of the final beer. Accounting for the increase in the fermentation cycle times, the capacity of the existing brewing plant is increased by 20-30%. Although the productivity of individual fermentation is increased, there is an increase in the fermentation cycle time and a decrease in the total number of fermentations per unit time and per fermentation vessel. Other disadvantages are associated with high osmotic pressure at the beginning of the fermentations, nitrogen limitation, high carbon dioxide concentration and high osmotic pressure towards the end of the fermentations and as a result of the above factors, sluggish and incomplete fermentation. This is especially critical point considering that industrially brewer's yeast is reused several times in beer fermentations. High gravity brewing also reduces the cycles in which yeast can be used in subsequent fermentation rounds. Desired trends for brewer´s yeast strains used in high gravity brewing are stress resistance, the ability to achieve faster and more complete fermentation compared to controls, higher viability

at the end of the fermentation process, and flavor profiles and flocculation behavior similar to those obtained from brewing at lower gravities. A common method to increase the wort concentration without need for modifying the existing brewing capacities is by the use of syrups as liquid adjuncts. Normal 12°Plato wort requires a minimum free amino nitrogen concentration of 160 mg L^{-1}. However, there is a risk that the use of such syrup dilutes the available nitrogen concentration of the finished wort and results in beer with elevated levels of acetate esters and higher alcohols. To overcome this challenge, the high gravity beer fermentations at > 18°Plato need to be supplemented either with a sufficient amount of free amino nitrogen (FAN) of at least 280 mg L^{-1}., or the fermentations should be performed under semi-aerobic conditions (87). The free amino nitrogen value represents the amount of nitrogenous compounds present in the wort - amino acids, ammonia, small peptides and terminal α – amino nitrogen groups of bigger peptides and proteins. While most of these compounds are utilized by brewer´s yeast, the disadvantage of the FAN method is that it also partially measures proline, amino acid that cannot be utilized by the brewer´s yeast. FAN must be sufficiently high to ensure that lack of nitrogenous compounds is not limiting the fermentation. Thus, with the increase in the gravity, higher amounts of FAN are needed for achieving efficient fermentation.

Research has been focused on studying the yeast physiological state in high gravity beer fermentations. It has been reported that after dilution to the desired alcohol content in some cases high gravity produced beer is lighter, smoother, less sweet and with more fruity, estery flavour compared to similar beer produced at normal gravity. Additionally, using maltose syrup instead of glucose syrup as a supplement for increased fermentation gravity provided a more desirable environment for the brewer´s yeast cells (88). Cells grown in very high gravity beer fermentations with maltose as the predominant carbohydrate compared to those grown in all malt high gravity wort with predominantly glucose or fructose, result in higher viability and vitality, higher intracellular trehalose content and with more favorable profile of the final beer - in terms of lower amounts of esters. Excessive concentration of esters in high gravity beer fermentation results in sweet, unpleasant flavors in the final beer. These observations were further confirmed by recent study from our own lab also focusing on studying the brewer´s yeast stress response to high gravity beer fermentations. Fermentations with the popular lager beer yeast strain Weihenstephan 34/70 were performed at average gravity of 14°P and at high gravity - 21° and 24°P. The higher gravity was achieved by the addition of glucose or maltose syrups to the basic wort. Higher viability and vitality and higher intracellular trehalose content was observed for the brewer´s yeast cells grown in maltose supplemented wort compared to those grown in glucose supplemented wort. Thus, our results also confirmed that maltose syrup addition offers a more favorable environment for the brewer´s yeast cells. As the sugar syrups did not contain any free amino nitrogen, by increasing in the gravity, the FAN values were reduced. As a result of the increased carbon to nitrogen ratio, stuck and incomplete fermentation was observed at 24°P, especially pronounced in the fermentations with glucose syrup addition. After dilution to the desired ethanol content, the resulting beer from those fermentations had elevated levels of ethyl acetate and isoamyl acetate compared to beer with the same alcohol content produced from average gravity (Piddocke and Olsson, unpublished data).

Immobilized yeast cell technology

Traditionally beer is a batch process, which has been optimized in the last century by the transformation from open fermentation vessels to closed cylindroconical tanks with the ability to ferment large volumes of wort with a significant improvement in product quality and hygiene. Although the first continuous fermentation system appeared in the 1960s, because of a number of limitations, only a few systems have appeared on large beer production scale up to date. Such challenges are increased risk of contamination as the wort had to be stored in supplementary holding tanks, variations in beer flavor, complex system design and a lack of flexibility. The most important disadvantages in the use of the immobilized yeast include restricted mass transfer and therefore restricted availability of nutrients, particularly oxygen, and limitations in the removal of potentially toxic products such as ethanol and carbon dioxide. As a result of the poorer mass transfer, yeast cells have lower specific oxygen uptake and growth rate, and consequently slower and insufficient amino acid uptake. Such fermentation results in beer with less than desired ester content. Reduced water activity has also been observed for immobilized yeast cells. The use of immobilized yeast for beer production has its advantages in terms of improved cell stability, higher conversion rates, faster fermentation rates, enhanced volumetric productivities, easier implementation of the continuous operation, and improved cell recovery and reuse (89). The later is particularly important for high gravity beer fermentations as cell cycle and reuse is an issue. The high volumetric efficiency is obtained by increased yeast cell concentrations in the reactor, compared to traditional batch systems. There is evidence suggesting that the physiology of immobilized yeast varies from that of the free cells. Comparison between the metabolic activity of immobilized and free cells has shown an activation of the yeast energy metabolism and an increase in storage- glycogen and trehalose and structural- glucan and mannan polysaccharides (89). The glucose uptake rate and the yield of ethanol has been shown to be increased, the intracellular pH is lowered and alterations in the activities of glycolytic enzymes has been observed as well. The increased resistance to starvation in the immobilized yeast cells is associated with improved ethanol tolerance. A major challenge for application of immobilized yeast in industrial food fermentation is finding inexpensive, stable, reusable and nontoxic carriers which also allow cell growth with a minimum of mass transfer limitations. A single example of successful implementation of continuous beer production process in the 1960s was achieved by Morton Coutts at Dominion Breweries in New Zealand. With the development of new immobilized technologies in the 1970s, more continuous beer fermentation systems have been developed as well. Presently, commercial scale yeast immobilized reactors are used for the maturation of the final beer in a conventional brewing process or for the production of alcohol free beer. Two continuous industrial maturation systems exists in Finland - one at Sinebrychoff Brewery, with capacity of 1 million hl per year and the other system at Hartwall Brewing developed with Alfa Laval and Schott Engineering. Both systems consist of separator, which prevents the growth of yeast in the next stages, a heat treatment unit, accelerating the conversion of α-acetolactate to diacetyl and acetoin, and a packed bed reactor with yeast immobilized on DEAE-cellulose granules or porous glass beats, respectively, helping for the further removal of the remaining diacetyl. To maintain product quality for both systems, strictly anaerobic conditions should be followed through the whole process. Currently, the heat treatment is

replaced with enzymatic treatment taking place in a fixed bed reactor. In this method α-acetolactate decarbohylase are immobilized on multilayer capsules, but still the further reduction of diacetyl takes place in a second packet bed reactor (90).

Besides maturation of the final beer, the use of immobilized yeast in brewing has the advantage of achieving broader commercial application in the production of alcohol free beer. The key for alcohol free fermentation is still to utilize the carbonyl compounds during the fermentation, but keep as little flux towards ethanol as possible. Bavaria brewing in the Netherlands has been using a packed bed immobilized reactor with a capacity of 150 000 hl annually for producing alcohol free beer (91). This system also uses yeast immobilized on DEAE-cellulose located in a specifically designed vessel called "Immocon" reactor. In this process wort with low fermentability is used and is boiled extensively in order to reduce the concentration of volatile aldehydes and carbonyls. Following the cooling, the wort is acidified with lactic acid produced by a separate bioreactor containing immobilized bacteria *Lactobacillus amylovorus*. The aerated wort is maintained at a temperature of 12 °C for 24 hours, promoting yeast growth, followed by a period with the wort at 4 °C and further increase in the flow rate with a final period at a temperature of 0 to 1 °C. The low temperature and anaerobiosis ensures limited fermentation without significantly affecting the reduction of the carbohyl compounds by the yeast. The final ethanol concentration from such fermentation does not exceed 100 µg/L. Although esters and higher alcohols in such fermentation are present in lower concentrations than in lager beer produced by conventional methods, this system is used worldwide with reported annual production of 700 000 hl (92). As glucose is constantly present in the media, maltose and maltotriose concentrations remain unchanged. The Japanese brewery Kirin implemented a three stage 100 hl pilot plant continuous fermentation system. The development of the system was based on the assumption that yeast of the same physiological state will produce beer with the same quality (93). The first stage of the process used stirred tank for the "batch" phase of the continuous fermentation process. The used yeast is inoculated in a media with desired free amino nitrogen concentration. As a result of the yeast´s growth, FAN concentrations are reduced, higher alcohols are produced and pH values are decreased. In the second stage, ethanol and esters are produced anaerobically. As an improvement to the system, the Ca-alginate gel beads used as a carrier were replaced with ceramic beads which increased the longevity and viability of the yeast. However, for scaling up the system in the second stage, the company faced problems associated with efficient cooling of the fermentation vessel. To overcome these difficulties, cooling pipes were inserted in the packed bed reactor. The third stage takes place in a packed bed maturation column. This last stage is a very important part of the process as immobilized yeast fermentation results in higher vicinal diketones concentrations. The whole brewing process in this three stage continuous beer fermentation system takes from 3 to 5 days.

It has also been suggested that the best primary fermentation can be achieved by an immobilized yeast reactor followed by a stirred fermentor containing free suspended cells (92). This approach would allow better control of the ester production since higher alcohol formation may proceed in the immobilized reactor via the biosynthetic route, but with limited amino acid uptake. In the later stage of the stirred fermentor, amino acid assimilation will occur and provide substrates for

esterification using the already present higher alcohols and ethanol. There is currently no production scale implementation for primary fermentation using immobilized yeast, but a few systems have achieved pilot scale trials. One of them uses a three stage system, where the first vessel consist of aerobic stirred fermentor maintained at 13°C with a 100 hl working volume. Yeast growth and production of higher alcohols is promoted (94). In the second stage two linked immobilized static yeast reactors of 100 hl each and ceramic bead carriers are used. Because of the lack of mixing and high rates of heat production from the primary fermentation, a high cooling capacity is needed to maintain the temperature at 8°C. The third vessel has a similar design to the second one and there, the maturation of the green beer takes place. The total residence time was three to four days and the resulting beer showed significant differences in its characteristics compared to the conventionally produced beer in terms of higher ethanol concentration, higher pH and sulfur dioxide concentrations as well as elevated bitterness and lower levels of higher alcohols. Additionally, this system faced a challenge during the six months pilot plant trials due to an inability to obtain a steady state, resulting in continuously changing performance.

In a different system (95), two stages of continuous primary fermentation were used and the yeast was immobilized on silicon carbide. In the first reactor the in-flowing wort is circulated through a silicon carbide matrix, before being transferred to the second vessel. The second vessel has a cylindroconical design and contains suspended cells. An external circulation loop controls the mixing and the temperature is controlled by in-line heat exchangers. It was possible to obtain a steady state condition using this system and the resulting beer, both lager and ale, showed profiles similar to conventionally brewed beers. Fermentation trials at 24°C with top fermenting ale yeast achieved a residence time of 8 hours.

Immobilized cell technology has the potential of offering an advantage to the brewing industry in terms of reduced fermentation time without reduction product quality. However, to commercialize its use on an industrial scale, there is a need for further investigation of the immobilized yeast physiological and metabolic properties which may influence the flavor profile of the final product (96). Further improvements to the immobilized yeast bioreactors involve research and a more in-depth understanding of the immobilization mechanism, finding cheaper and more stable carrier materials and improved reactors in terms of cooling and energy efficiency.

Brewing in the post genomic era

With the availability of the *S. cerevisiae* genome sequence together with bioinformatics tools which enable integration and interrogation of large *x-omics* data sets, it is possible to identify high-probability targeted genetic strategies to increase yield, titer, productivity, and/or robustness of existing industrial process (34). For the last ten years the number of publications for *x-omics* characterisation of *S. cerevisiae* or other already sequenced organisms used for many industrial processes has increased tremendously. Due to the complex genome nature of brewer´s yeast, associated with its polyploid nature and chromosomal translocations, its sequencing is challenged. This is also the main reason that the *x-omics* characterisation of the brewer´s yeast compared to those of the common *S. cerevisiae* is behind. Despite the mentioned limitations of the

characterisation of the transcriptome, proteome and metabolome of the beer fermentation process, it is possible to elucidate key metabolic pathways and to determine their constraints and regulative influences and use it for further process development. *X-omics* is a general term referring to collection and analysis of any global data set whereby any type of informational pathway with reference back to the cell´s genome is investigated. Today, the available "*x-omics*" techniques as system biology tools are also used to determine the nature of the genetic perturbations leading to the desired phenotype from a strain obtained from a random or directed mutagenesis or through evolution. Such an approach is described as reverse metabolic engineering (34).

Transcriptome

Transcriptomics is the study of the mRNA expression levels on the genome wide basis for a population of cells from specific environmental conditions (34). For the last ten years with the availability of the *S. cerevisiae* sequenced genome and the first commercial Affymetrix microarray data platform, large number of laboratory and hybrid *S. cerevisiae* strains have been extensively studied during growth on rich, or limited media, anaerobiosis, exposure to oxidative or ethanol stress, low pH, low temperature, high gravity fermentations, etc. (12). Comparison of the data from such a study with transcriptome data based on laboratory *S. cerevisiae* yeast strain grown in, for example, YPD media, showed both similarities and significant differences. The main differences will be influenced by the different physiological behavior and by the different ploidity of the brewer's and laboratory yeast cultivated in the same media.

Although these studies offer a significant amount of valuable information, it is of utmost importance for the brewing industry to study the complexity of the brewer's yeast transcription profile in its native environment, during authentic beer fermentation. Overall, several studies on the transcriptional responses of brewer´s yeast have been reported, using different strains, under different media conditions, and on a scale from 3L laboratory scale vessels to 5000 hl full scale cylindroconical fermentors (12).

Transcriptome samples collected from early exponential and stationary phase (97, 98) during 2L scale fermentation trials revealed high expression during the first two days of the fermentation on genes involved in glycolysis, fatty acid and ergosterol biosynthesis, protein biosynthesis, chaperone function and protein folding. 71% of the genes expressed in this period had well defined function but during the third day of the fermentation high numbers of genes with presently unknown function were expressed. Most of the genes were up-regulated between 24h and 48h of fermentation and their expression decreased with the progress of the fermentation. Although stress response genes were expressed throughout the fermentation, their highest expression was noticed at the initial stage of the fermentation and at the peak of the alcohol formation. A complete repression of many stress response genes and genes involved in protein synthesis has been observed through the eighth day of fermentation (97). It has also been demonstrated that there are at least several ORFs with significantly different transcript levels at the different stages of the beer fermentation with unknown function.

Further, genome wide expression analyses were used successfully in identifying environmental factors and conditions important for industrial beer production. Study of industrial lager yeast strain, grown in zink deficient and in zink repleted media, revealed the importance of two homologues genes- *YOR387c* and *YGL258w* as molecular markers for determing zink deficiency in yeast (99). Similar gene expression patterns observed for *ERG10* and *TRR1* over the first 24 hours of the fermentations also lead to the hypothesis for possible interactions between ergosterol biosynthesis and oxidative stress response. Further transcriptome analysis showed that the deletion mutants for the ergosterol biosynthesis pathway have high sensitivity to oxidative stress. Metabolic activity assay of the mutants also proved that ergosterol is an important factor in restoring the fermentative capacity of the yeast cells after storage (100).

Comparative analysis of the transcriptional response for laboratory and lager brewer´s yeast exposed to saline stress has also been done (101). Both strains showed faster response of gene expression in low saline concentrations compared to high saline concentrations. At high saline concentrations, genes encoding enzymes involved in the carbohydrate metabolism and energy production were up-regulated for both strains. For the brewer's yeast also the genes involved in the amino acid metabolism were up regulated under high saline concentration. Genes encoding sodium ion efflux pump and copper metallothionein proteins were higher expressed in the brewing strain compared to the laboratory strain. Based on the obtained transcriptome data, candidate genes for construction of improved saline stress tolerant strains were suggested. DNA microarrays has also been used to study the genes responsible for superior fermentation performance in high gravity beer fermentations (> 22°P) of UV - mutagenized clones of the industrial lager strain CMBS 33 (32). Ten genes showed differential expression in the variants compared to the control strain. Among those, the highest change in expression was observed for *HXK2*, whose expression was reduced by two fold and *LEU1* and *ARG1* with expression reduced from two to six fold.

In all of the above examples, DNA microarrays based on the *S. cerevisiae* S288C genome have been used to study the transcriptional responses in brewer´s yeast. The genes derived from the *S. cerevisiae* part of the genome are nearly 100% identical to the published sequence of the *S. cerevisiae* S288C genome. In order to study the sequence similarity, several genes from the non-*S. cerevisiae* part of the genome have been cloned and sequenced and have been identified as 75-85% identical to the S288C genome. Thus the limitations of using *S. cerevisiae* DNA arrays for studying lager the brewer's yeast transcriptome profile are associated with an inability to reveal differential expression, as the Sc-type of the lager brewing genome will preferentially hybridize to the arrays mRNA from the non-*cerevisiae* part of the genome. And it will be either measured as a part of the expression coming from the *S. cerevisiae* part of the genome or if very low similarity occurs it will be undetectable. Besides these limitations such studies still provide a solid base for studying the genetics and physiology of the lager brewing yeast strains under controlled environmental conditions (12).

To overcome the problems with the complexity of the lager brewer´s yeast genome and accomplish complete genome wide expression studies, several alternative approaches on the common DNA array technology have been used. Massively parallel signature sequencing has been used to study

the expression profile of nearly 1400 homoeologues in beer fermentation (13). The authors showed that almost half of the homoeologues showed differential expression and thus emphasized the need for using DNA arrays containing all of the lager brewer´s yeast ORFs for subsequent studies of the transcriptome profile in beer fermentations.

In a different study a random genomic library of the bottom fermenting lager yeast strain Weihenstephan 34/70 was developed. With DNA fragment size of around 2,5 kbp, more then 50 000 independent clones has been obtained and in total 20 160 clones were printed on shotgun DNA microarray. In the shotgun strategy, the genome is represented by random DNA fragments of unknown sequence arrayed on a glass slide. DNA fragments that contain regulated genes are identified by differential expression with two labeled cDNAs. The possibility of putting thousands of gene sequences into a single slide allows the use of shotgun clones in order to proceed with microarray analysis without a completely sequenced genome (102).

Transcript analysis with the aid of the affinity capture (TRAC) method has been used to follow the expression of 70 selected genes involved in metabolic pathways such as maltose, glucose, glycerol and lipid metabolism, amino acid biosynthesis, flavor and aroma compound formation and flocculation at a frequency of up to 10 times per day (103). In this method the mRNA of interest is hybridized to a gene specific probe pool, containing oligonucleotides of the genes of interest with double fluorophore label and biotinylated oligo (dT) probe. The hybridized targets are immobilized with affinity capture to streptavadin coated magnetic beads. In the next step the unbound sample material is washed out, followed by the elution of the probes from the targets and further identification and quantification using capillary electrophoresis (104). The authors observed rapid changes in expression during the first 2 to 6 hours of fermentation for genes associated with maltose metabolism, glycolysis and ergosterol synthesis. During the final fermentation stages unexpected up-regulation of the genes involved in oxygen requiring pathways was observed. For five of the genes the expression of *S. cerevisiae* genes and *S. bayanus* genes, both present in the lager yeast genome were studied. The probes for the *S. bayanus* genes were based on the *S. bayanus* sequenced genome and designed to specifically recognize them. In the cases of genes *MALx1*, *ILV5*, *ATF1* the expression profile showed very a similar profile, while in the case of *ADH1* and *ERG3* the expression profiles showed significant difference depending on if they originated from *S. cerevisiae* or *S. bayanus*.

Even though the genome sequence of the lager brewer's yeast is challenged by the fact that it is tetraploid or allotetraploid in its nature and that chromosomal rearrangement occurs with a different frequency and under different environmental conditions, an attempt to sequence the genome of the lager brewer´s yeast strain has been made (13). Using the genome sequence of the lager strain Weihenstephan 34/70, DNA microarrays using the Affymetrix platform and containing both Sc type genes and the non-Sc type genes have been constructed. The lager brewing yeast array (LBYG) contain 6 150 Sc type ORFs and 5597 non- Sc ORFs. Additionally, the array contains 28 mitochondrial ORFs of non- Sc type, ORFs that have weak homology to genes submitted in the data bases and ORFs longer that 150 bp in inter-genic sequences. Such an array is especially valuable when specific metabolic pathways are studied and are known to be influenced predominantly by the

non-Sc part of the lager brewer´s yeast genome, as, for example, is sulfite production in lager brewing (105). As sulfite production is greatly influenced by the oxygenation conditions, fermentation trials at different oxygenation conditions have been tested. While the expression of the Sc genes remains unchanged, higher expression levels of the non-Sc genes was reported for all oxygenation conditions compared to the non-oxygenated condition. The expression of the Sc genes also decreased significantly when the yeast was propagated anaerobically. Genomic hybridization of several lager beer yeast genomes revealed that the genomic differences among the lager strains occurred not only in intra-/inter-genic sequences but also in the number of chromosomes. The authors suggested a possibility that those differences might be induced by the oxygen stress.

As shown in the above studies, where analysis of transcript levels in brewer´s yeast has been performed, the technology offers a wealth of information. It is of further significant importance for the development of transcriptome analysis to also include information on the differential regulation of genes from the Sc or non-Sc parts of the genome. As transcriptome analysis in such hybrid strains as lager brewer´s yeast is a very challenging task, substantial care has to be taken to ensure that the measurements of the mRNA expression levels reflect the real representation of the state of the cells. Consequently, both the array technology and the data treatment have to be further developed to properly address these issues.

Proteome

Proteomics is the physical and biochemical study of all proteins coded by ORFs in an organism (106). Proteomics is one of the "-omics" tools being challenged by the fact that the required sensitivity and accuracy of the analytical methods necessary for measurement of all proteins has been developed more recently than the analytical methods used in the other -omics analyses (34).

Despite that proteome analysis has been successfully used for determining further strain improvement and metabolic engineering strategies, as well as improvement of the design and control of different industrial fermentations of *S. cerevisiae* (34), the proteome research reported to date on brewer´s yeast is limited. The first study on proteome analysis of brewer's yeast also proved the complexity of the genetic nature of the brewer's yeast (107). The authors showed that the proteome of lager brewing yeasts can be interpreted as a superimposion of two elementary proteomes - one corresponding to proteins encoded by a *S. cerevisiae*- like genome and the other corresponding to a divergent *Saccharomyces* species group, with closest representation in the *S. pastorianus* strain. A map of industrial lager brewer's yeasts containing proteome has been established containing the individual origin of proteins and with identification of protein spots by comparison to known *S. cerevisiae* proteins (107). Comparison of the later developed protein map of ale fermenting yeast to the protein maps of lager brewer's yeast and *Saccharomyces cerevisiae* strain S288c confirmed that on the proteome level, the ale strains were much closer to the S288c than the lager yeast strains. In a global study, 30 lager brewing yeast proteins were newly identified using matrix assisted laser desorption/ionization – time of flight (MALDI-TOF), tandem mass spectrometry (MS/MS) and database searches against the protein sequence of *Saccharomyces*

cerevisiae (108). The identified proteins, unique for brewer's yeast strains, corresponded to proteins which did not co-migrate with known proteins of *S. cerevisiae* separated on 2-D gels. Two-dimentional gel electrophoresis has also been used to identify proteins induced during the lag and early exponential phase of lager brewing yeast cultivated in minimal medium (109). Proteome analysis has also been used to study the dynamics in the ale brewer's yeast proteome for several generations (re-pitching cycles). In the first generation most changes were related to the transfer from aerobic propagation to anaerobic growth in fermentation. More changes were observed by increasing the number of generation cycles, but some stress related proteins such as Hsp26p, Ssa4p and Pnc1p have been constitutively expressed throughout the generations (110). The high complexity of the brewer's yeast genome with sequence not completely revealed also reflects the complexity in their proteome picture. As the whole DNA sequence for brewer's yeast is not available, not all encoded protein sequences are known. Yet, today´s available tools for proteome analysis presents promising opportunities for the identification of brewer's yeast proteins which are homologous to the corresponding *S. cerevisiae* proteins.

Metabolome

Metabolomics is an appealing tool for quantitatively characterizing the relationship between the genome and phenotype of the cells and providing an important complement to the mRNA and protein measurement when studying cellular function (34). While the concentrations of flavor and aroma compounds are routinely reported in physiological studies on brewer´s yeast for different strains and conditions, reports on global metabolome studies of brewer´s yeast are very limited. Metabolome footprinting in combination with genetic fingerprinting by amplified fragment length polymorphism (AFLP) and comparative genome hybridization (CGH) has been used to distinguish nine brewer's yeast strains, among which were two ale, six lager and a reference *S. cerevisiae* strain (15). Metabolic footprinting monitors the exometabolome, which in turn is a combination of the metabolites secreted from the intracellular volume and any unused growth medium components (111). Direct injection electrospray mass spectrometry (DIMS) and gas chromatography-time of flight-mass spectrometry (GC-TOF-MS) were used to assess the possibility of differentiation of the nine brewer's yeast strains based on their metabolome footprinting and subsequent multivariate analysis. The metabolic footprinting revealed a wide variety of metabolites including amino and organic acids, carbohydrates, lipids, alcohols and phosphorylated compounds. The results of this metabolome study showed a similar, but not identical pattern of diversity compared to the AFLP, but suggested a clear separation between the lager and ale yeast strains. Both the metabolome profiling and AFLP showed that although originating from different geographic regions, four out of the six lager strains demonstrated close similarity and probable origin from a similar brewing strain. One of the lager strains showed distinct differences in the metabolic and genetic profile, while others showed metabolic diversity but genetic similarity to the control *S. cerevisiae* and one of the ale strains. Metabolic footprinting has been especially valuable in the case of the two ale strains, as genetically they are clustered with other ale strains, but metabolically they clustered with each other. The metabolites showing concentration related differences between those two ale and the

other strains included trehalose and organic acids such as octadecanoic, lactic and *trans*-aconitic. As trehalose plays a role of protectant, contributing to the survival of yeast under various stressful conditions, it has been suggested that this metabolite may be useful in discrimination between strains. The results proved the discriminatory effect of the metabolome analyses but also emphasized the need for combination of both approaches for full and complete strain characterization.

Recent work from our laboratory examined the intra- and extracellular metabolome profile from the early exponential and stationary phase of average - 14°Plato and high gravity - 21° and 24°P beer fermentations carried out with two ethanol tolerant and one less ethanol tolerant commercially used lager beer yeast strains. High gravity was achieved by adding glucose or maltose syrup to the basic wort. Samples from the early exponential phase from the average gravity fermentations and those collected from the high gravity fermentations revealed very similar metabolite profiles among the three strains, while the samples from the stationary phase showed significant differences in the metabolome profiles of the less ethanol tolerant and from the well ethanol tolerant strains. Additionally, for the less ethanol tolerant strain there was clear distinction in the metabolome profile between the samples collected from the stationary phase for the fermentations with glucose syrup supplementation versus the fermentations with maltose syrup supplementation. The compounds that contributed to this separation were mainly TCA intermediates and amino acids mostly associated with the pyruvate and phosphoenolpyruvate metabolism. The metabolite profiling clearly demonstrated the ability to distinguish between different fermentation conditions and increased energy consumption by the cells exposed to more severe stress in the high gravity wort, achieved with glucose supplementation, reflected the metabolome profile with increased intracellular concentration of the puruvate and related metabolites and confirmed stuck and incomplete fermentations with high concentration of the intermediate compounds from the TCA cycle.

These results also supported the application of metabolome analysis as a useful and promising tool for determining the “momentum” physiological state of the yeast cells in the course of beer fermentation. Furthermore, the information could be used for identification of further targets for metabolic engineering or process optimization strategies, but also to understand the fundamental changes occurring after subtle genetic or process perturbations.

The future perspectives in the beer fermentation process optimization

The focus of this review has been in three different directions. First and most important, to present a summary and follow the historical development of studies on the lager brewer’s yeast genome and beer fermentation as such, through its optimization with the development of relevant genetic and metabolic engineering strategies and to continue in the post genomic era with the development and implementation of the “*x-omics*” tools. Secondly, the review comments on two of the main process optimization strategies implemented in laboratory and industrial beer production scale today- high gravity brewing and continuous beer fermentation with the use of immobilized yeast and to discuss

the benefits of those processes and the challenges they offer. To a lesser extent this review was also focused on summarizing the use of the metabolic engineering and molecular biology tools for barley and hops improvement.

Despite the complexity of the beer fermentation and the brewer´s yeast genome, this review demonstrated that various classical methods and metabolic engineering strategies have been successfully applied for obtaining brewer's yeast strains with improved sugar utilization, increased ethanol yield, reduced off-flavor formation and enhanced desired flavor formation. Despite the existence of obviously successful metabolic engineering strategies resulting in strains with enhanced product characteristics, due to the negative public perception and the complex legislation, beer producers as well as the other food biotechnology producers are reluctant to use GMO strains. Thus, recognized by the public and governmental legislations and proven to be successful, mutagenesis remains today the commonly used genetic tool for brewer´s yeast strain improvement. While one can argue that mutations do not provide any mechanical information about the achieved perturbations, others defend that desired mutations found in isolates after for example random mutagenesis, compared to mutations obtained through metabolic engineering, are usually more subtle and lead to more balanced performance. For improved hops varieties, another alternative has been the use of molecular markers to target specific traits.

Yet, the situation with the use of GMO strains in brewing is now changing. Legislation in some countries (78) does not consider self-cloned organisms as GMO organisms and thus allows for the use of self-cloned organisms in the beer production process. On the other hand, although GMO strains are not directly used for beer production, the use of enzymes, usually a product of GMO strains is now recognized by the authorities, accepted by the brewing communities and applied in industrial beer production beer producers. Thus, today´s enzymes are of essential help when malt quality and price is an issue and alternative raw materials start to emerge on the market.

The combination of genetics and a wide variety of *x-omics* data such as transcriptomics, proteomics and metabolomics can be applied in metabolic engineering and/or in process optimization strategies to identify new targets for improved phenotype and further process development. However, to have the development efforts focused on it and to be broadly applied in bioprocess development, especially in the case of the low- value, low cost products as beer, it is essential that the "*omics*" technologies are implemented on high-throughput and easy to use platforms (34). The high competitiveness of the beer market is driving the producers to develop processes resulting in higher yields and productivity where efficiency, convenience, added value, cost reduction and innovation are the keys. As the margins and areas for improvement are narrowing, they can only be satisfied with innovative approaches that might yet be undiscovered. The great advantage of *x-omics* analyses is their ability to predict and answer questions like the type of regulation (transcriptional or translational) for specific metabolic pathways or the distribution of carbon through the different metabolic pathways. Thus, to some extent the implementation of the "*x-omics*" analyses could be an alternative to the direct metabolic engineering strategies. For example, if we know that certain perturbation is not transcriptionally but metabolically regulated, other means than metabolic engineering strategies could be used. Such strategies as modification of wort compositions, enzyme

addition or fermentation process optimization might be the way to achieve the desired phenotype in the given environment.

Brewer´s yeast fermentation is a major part of the whole beer production process. Besides brewer´s yeast fermentation optimization, today's brewing industry is focused on continuous process optimization. Efforts are focused towards reduced energy and water usage, reduced volume and strength of waste water discharge and identifying options to recover and capture carbon dioxide from the fermentation process (112). In the present competitive beer market where high quality and low price is demanded, high gravity brewing is the attractive approach offering both substantial economic savings, in terms of water and energy supply, and increased volume without the need for expansion of existing brewing plants. The use of immobilized yeast in continuous secondary fermentation is also a desired trend which offers substantial reduction in the maturation time, resulting again in larger volumes of beer produced per unit time and savings mostly in terms of energy.

But for successful implementation of any of the brewing optimization strategies it is important to change the public perception and policy and garner public, governmental and corporate support. It is essential for new technologies to succeed and they in turn are needed for beer and brewing process development.

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References

1. W. Kunze, Technology Brewing and Malting, VLB Berlin, Verlagsabteilung, 1999, pp19-26.
2. C. Boulton and D. Quain, *Brewing Yeast and Fermentation*, Blackwell Science Ltd., Oxford, 2006, pp. 5-15.
3. D. E. Briggs, C. A. Boulton, P. A. Brookes and R. Stevens, *Brewing Science and Practise*, Woodhead Publishing Limited, Cambridge, 2004, pp. 363-366.
4. Plato Logic Limited, World beer report 2007, Available at http://www.platologic.co.uk/, Accessed February 29, 2008.
5. E. Pajunen and A. Hummer, Proceedings of the 31st European Brewing Convention Congress, Venice, 2007, pp. 1496-1509.
6. D. E. Briggs, C. A. Boulton, P. A. Brookes and R. Stevens, *Brewing Science and Practise*, Woodhead Publishing Limited, Cambridge, 2004, pp. 1-10.

7. G. Walker and P. van Dijck, in A. Querol and G. H. Fleet, eds., *The Yeast Handbook*, Springer-Verlag, Berlin Heidelberg, 2006, pp. 111-152.

8. J. Hansen and M. Kielland-Brandt, J.H. de Winde, ed., *Topics in Current Genetics*, vol. 2, Springer-Verlag, Berlin Heidelberg, 2003, pp.143-164.

9. C. Boulton and D. Quain, *Brewing Yeast and Fermentation*, Blackwell Science Ltd, Oxford, 2006, pp. 29-46.

10. J. Hansen and M. Kielland-Brandt, In: F.K. Zimmerman and K.D. Entian (eds.), Yeast sugar metabolism, biochemistry, genetics, biotechnology and applications. Technomic Publishing, New York, 1997, pp. 527-559.

11. S. Dequin, Appl. Microbiol Biotechnol. **56**, 577-588 (2001).

12. K. Smart, Yeast **24**, 993-1013 (2007).

13. Y. Kodama, M. C. Kielland-Brandt and J. Hansen in P. Sunnerhagen and J. Piškur (eds.), *Comparative Genomics*, Springer- Verlag, Berlin Heidelberg, 2005, pp. 145-164, DOI 10.1007/b106370.

14. M.T. Fernandez-Espinar, E. Barrio and A. Querol, Yeast, **20**, 1213-1226 (2003).

15. G. Pope, D. MacKenzie, M. Defernez, M. Aroso, L. Fuller, F. Mellon, W. Dunn, M. Brown, R. Goodacre, D. Kell, M. Marvin, E. Louis and I. Roberts, Yeast, **24**, 667-679 (2007).

16. E. C. Hansen, Compt Rend Trav Lab Carlsberg, **7**, 179-217 (1908).

17. Y. Tamai, T. Momma, H. Yoshimoto, Y. Kaneko, Yeast, **14**, 923-933 (1998).

18. H. Yamagishi and T. Ogata, Syst. Appl. Microbiol., **22**, 341-353 (1999).

19. S. Casaregola, H. V. Nguen, G. Lapathitis, A. Kotyk and C. Gaillardin, Int. J. Syst. Evol. Microbiol, **51**, 1607-1618 (2001).

20. M. B. Pedersen, Carlsberg Res. Commun. **51**, 163-183 (1986a).

21. M. B. Pedersen, Carlsberg Res. Commun. **51**, 185-202 (1986b).

22. J. Hansen and M. Kielland-Brandt, Gene, **140**, 33-40 (1994).

23. Y. Kodama, F. Omura, K. Miyajima and T. Ashikari, J. Am. Soc. Brew. Chem., **59**, 157-162 (2001).

24. L. Hoffmann, PhD thesis, University of Copenhagen, Copenhagen, (2000).

25. Y. Nakao, Y. Kodama, N. Nakamura, T. Ito, M. Hattori, T. Shiba and T. Ashikari, Proc. 29th Congr. Eur. Brew. Conv., Dublin, 2003, pp. 524-530.

26. M. Kellis, N. Patterson, M. Endrizzi, B. Birren and E. Lander, **423**, 241-254 (2003).

27. U. Bond, C. Neal, D. Donnelly and T. James, Curr. Genet. **45**, 360-370 (2004).

28. T. C. James, J. Usher, S. Campbell and U. Bond, Curr Genet **53**, 139-152 (2008).

29. F. Pizarro, F. A. Vargas and E. Agosin, Yeast, **24**, 977-991 (2007).

30. K. J. Verstrepen, F. F. Bauer, J. Winderickx, G. Derdelinckx, J-P. Dufour, J. M. Thevelein, I. S. Pretorius and F. R. Delvaux, Cerevisia, **26**, 89-97 (2000).

31. T. Sasaki, J. Watari, M. Kohgo, N. Nishikawa and Y. Matsui, J. Am. Soc. Brew. Sc. **42**,164-167 (1984).

32. L. Blieck, G. Toye, F. Dumortier, K. J. Vertsrepen, F. R. Delvaux, J. M. Thevelein and P. van Dijck, App. Env. Microbiol. **73**, 815-824 (2007).

33. A. Mizuno, H. Tabei and M. Iwahuti, J. Biosc. Bioeng., **101**, 31-37 (2006).

34. J. M. Otero, G. Panagiotou and L. Olsson, Adv. Biochem. Engin/ Biotechnol, **108**, 2007, pp. 1-40, DOI 10.1007/10_2007_071.

35. J. Polaina, *Applied Mycology and Biotechnology*, Vol. 2, Elsevier Science BV, Amsterdam, 2002, pp. 1-17.

36. Z. Liu, G. Zhang and S. Liu, J. Biosc. Bioeng. **98**, 414-419 (2004).

37. Y. Zhang, Z.-Y. Wang, X-P. He, N. Liu and B.-R. Zhang, Int. J. Food Microbiol., **123**, 18-24 (2008).

38. C. S. Park, Y. J. Park, Y. H. Lee, K. J. Park, H. S. Kang and U. H. Pek, MBAA Tech Quart, **27**, 112-116 (1990).

39. J. C. Steyn and I. S. Pretorius, Gene, **100**, 85-93 (1991).

40. C. J. L. Klein, L. Olsson and J. Nielsen, Microbiology, **114**, 13-24 (1998).

41. S. L. Alves, R. A. Herberts, C. Hollatz, L. C. Milleti and B. U. Stambuk, J. Am. Soc. Brew. Chem. **65**, 99-104 (2007).

42. B. U. Stambuk, M. A. da Silva, A. D. Panek, and P. S. de Araujo, FEMS Microbiol. Lett., **170**, 105-110 (1999).

43. E. K. Han, F. Cotty, C. Sottas, H. Jiang and C. A. Michels, Mol. Microbiol. **17**, 1093-1107 (1995).

44. V. Vidgren, L. Ruohonen and J. Londesborough, Appl. Environm. Microbiol. , **71**, 7846-7857 (2005).

45. Y. Kodama, N. Fukui, T. Ashikari and Y. Shibano, J. Am. Soc. Brew. Chem., **53**, 24-29 (1995).

46. M. Meilgaard, Tech. Quart. MBAA, **11**, 87-89 (1974).

47. K. Blomquist, M. L. Suihko, J. Knowles and M. Penttilä, Appl. Envir. Microbiol. **57**, 2796-2803 (1991).

48. C. Gjermansen, T. Nilsson- Tillgren, J. G. Petersen, M. C. Kielland-Brandt, P. Sigsgaard and S. Holmberg, J. Basic Microbiol., **28**, 175-183 (1988).

49. S. M. Mithieux and A. S. Weiss, Yeast, **11**, 311-316 (1995).

50. Y. Zhang, Z.-Y. Wang, X.-P. He, N. Liu and B.-R. Zhang, Int. J. Food Microbiol. **123**, 18-24 (2007).

51. E. Nevoigt, R. Pilger, E. Mast-Gerlash, U. Shmidt, S. Freihammer, M. Eschenbrenner, L. Garbe and U. Stahl, FEMS Yeast Res. **2**, 225-232 (2002).

52. J. Hansen and M. C. Kielland-Brandt, Nat. Biotech., **14**, 1587-1591 (1996), DOI:10.1038/nbt1196-1587.

53. D. E. Briggs, C. A. Boulton, P. A. Brookes and R. Stevens, *Brewing Science and Practise*, Woodhead Publishing Limited, Cambridge, 2004, pp. 691-694.

54. F. Omura, Y. Shibano, N. Fukui and K. Nakatani, J. Am. Soc. Brew. Chem. **53**, 58-62 (1995).

55. H. Tezuka, T. Mori, Y. Okumura, K. Kitabake and Y. Tsumura, J. Am. Soc. Brew. Chem. **50**, 130-133 (1992).

56. C. Liegeois, N. Meurens, C. Badot and S. Collin, J. Agric. Food Chem. **50**, 7634-7638, (2002).

57. A. Irwin, R. Barker and P. Pipasts, J. Am. Soc. Brew. Chem. **49**, 140-149 (1991).

58. J. Dufour, M. Leus, A. Baxter, A. Hayman J. Am. Soc. Brew. Chem. **57**, 138-144 (1999).

59. J. Hansen, S. V. Bruun, L. M. Bech and C. Gjermansen, FEMS Yeast Res. **2**, 137-149 (2002).

60. K. Verstrepen, S. D. M. Van Laere, B. M. P. Vanderhaegen, G. Derdelinckx, J-P. Dufour, I. S. Pretorius, J. Winderickx, J. M. Thevelein and F. R. Delvaux, Appl. Environm. Microbiol. **69**, 5228-5237 (2003).

61. D. Hirata, S. Aoki, K. Watanabe, M. Tsukioka, T. Suzuki, Biosci. Biotechnol. Biochem. **56**, 1682-1683 (1992).

62. M. Lilly, M. G. Lambrechts, I. S. Pretorius, Appl. Environm. Microbiol., **66**, 744-753 (2000).

63. I. S. Pretorius, Yeast, **16**, 675-729 (2000).

64. J. R. M. Hammond and K. W. Eckersley, J. Am. Soc. Brew. Chem., **90**, 167-177 (1984).

65. M. E. Penttilä, M. L. Suihko, U. Lehtinen, M. Nikkola and J. K. C. Knowles, Curr. Gen., **12**, 413-420 (1987).

66. L.G. Jensen, O. Olsen, O. Kops, N. Wolf, K.K. Thomsen and D. von Wettstein, Proc. Natl. Acad. Sci., **93**, 3487-3491 (1996).

67. M. Stratford, Yeast, **8**, 25-38 (1992).

68. K. J. Verstrepen, G. Derdelinckx, F. R. Delvaux, J. Winderickx, J. M. Thevelein, F. F. Bauer and I. S. Pretorius, J. Am. Soc. Brew. Chem. **59**, 69-76 (2001).

69. L. Domingues, A. A. Vicente, N. Lima and J. A. Teixeira, Biotechnol. Biopr. Eng., **5**, 288-305 (2000).

70. O. Kobayashi, N. Hayashi, R. Kuroki and H. Sone, J. Bacteriol. **180**, 6503-6510 (1998).

71. J. Watanari, Y. Tanaka, M. Ogawa, H. Sahara, S. Koshino, M. L. Onnela, U. Airaksien, R. Jaatinen, M. Penttilä and S. Keränen, Yeast, **11**, 211-225 (1994).

72. M. Linko, J. Biotech. **65**, 85-98 (1998).

73. S. Tingay, D. McElroy, R. Kalla, S. Fieg, M. Wang, S. Thornton and R. Brettell, The Plant J., **11**, 1369-1376 (1997).

74. M. Kihara, Y. Okada, H. Kuroda, K. Saeki, N. Yoshigi, and K. Ito, Mol. Breed. **6**, 511-517 (2000), DOI: 10.1023/A:1026535407570.

75. D. E. Briggs, C. A. Boulton, P. A. Brookes and R. Stevens, *Brewing Science and Practise*, Woodhead Publishing Limited, Cambridge, 2004, pp. 227-254.

76. T. Roberts, Proceedings of the Course on Novel Developments in Brewing, Heriot-Watt University, Edinburg, UK, 2006.

77. D. Schuller and M. Casal, App. Env. Microbiol. **68**, 292-304 (2005).

78. R. Akada, J. Bioc. Biotech. **94**, 536-544, (2002).

79. European commission, web information. Available at http://ec.europa.eu/food/food/chemicalsafety/additives/com2006_425_en.pdf, accessed April 1, 2008.

80. Novozymes A/S, web information. Available at http://www.novozymes.com/en/MainStructure/ProductsAndSolutions/Brewing/Brewing.htm, accessed April 1, 2008.

81. Danisco A/S, web information. Available at http://www.danisco.com/cms/connect/corporate/products%20and%20services/product%20range/enzymes/brewing%20and%20distilling/brewing_distilling_enzymes_en.htm, accessed April 1, 2008.

82. R. Cana, U. Schliessner, McKenna Long and L.L.P. Aldridge, EU regulation of genetically modified enzymes, 2005. Available at: http://crossborder.practicallaw.com/main.jsp, accessed 11 January, 2008.

83. K. Staer, Proceedings of the 31st EBC, Venice, 2007, pp. 608-618.

84. European commission, Annex II and III, Directive 2001/18/EC, available at http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32001L0018:EN:HTML, accessed April 1, 2008

85. http://www.biocatalysts.com/pdf/technical_bulletins/TB104_Brewing.pdf

86. A.I. Whitear and D. Crabb, High gravity brewing- concepts and economics, *The Brewer*, **63**, 60-63, (1977).

87. C. Boulton and D. Quain, 2006, *Brewing Yeast and Fermentation*, Blackwell Science Ltd., Oxford, 2006, pp. 60-63.

88. O. S. Younis and G. G. Stewart, J. Am. Soc. Brew. Chem. **57**, pp. 39-45 (1999).

89. P. J. Verbelen, D. P. De Shutter, F. Delvaux, K. J. Verstrepen, F. R. Delvaux, Biotech. Lett. , **28**, 1515-1525, (2006).

90. F. Nitzsche, G. Höhn, R. Meyer-Pittroff, S. Berger, R. Pommersheim, Proc. of the 28th Europ. Brew. Conv. pp. 486- 494, (2001).

91. van Dieren, B., EBC Symp., Monogr. XXIV, Espoo, Finland, Verlag Hans Carl Getränke-Fachverlag, pp. 66-76, (1995).

92. C. Boulton and D. Quain, 2006, *Brewing Yeast and Fermentation*, Blackwell Science Ltd., Oxf., 2006, pp. 347-369.

93. T. Inoue, 1995, Proc. 25th Eur. Brew. Conv., pp. 25-36, (1995).

94. Y. Yamauchi, T. Kashihara, H. Murayama, et al., (1994), Tech. Qart. Mast. Brew. Assoc. Am., **31**, 90-94, (1994).

95. M. Andries, P. C. van Beveran, O. Goffin, P. Rajotte and C. A. Masschelein, Tech. Quart. Mast. Brew. Ass. Am., **34**, 119-122.

96. Biotechnology of Brewing New Technologies, web information. Available at: www.ecm.auckland.ac.nz/course/650361/Lecture3, Accessed February 29, 2008.

97. T. C. James, S. Campbell, D. Donnelly and U. Bond, J. Appl. Microbiol. **94**, 432-448, (2003).

98. K. Olesen, T. Felding, C. Cjermansen and J. Hansen, FEMS Yeast Research. **2**, 563-573 (2002).

99. V. Higgins, P. Rogers and I. Dawes, Appl. Env. Microbiol. **69**, 7535-7540 (2003).

100. V. Higgins, A. Beckhouse, A. Oliver, P. Rogers and I. Dawes, Appl. Env. Microbiol. **69**, 4777-4787 (2003).

101. T. Hirasawa, Y. Nakakura, K. Yoshikawa, K. Ashitani, K. Nagahisa, C. Furusawa, Y. Katakura, H. Shimizu and S. Shioya, App. Microbiol. Biotechnol. **70**, 346-357 (2006).

102. N. Kobayashi, M. Sato, S. Fukuhara, S. Yokoi, T. Kurihara, J. Watari, T. Yokoi, M. Ohta, Y. Kaku and T. Saito, J. Am. Soc. Brew. Chem.., **65**, 92-98 (2007).

103. J. Ratio, A. Huuskonen, H. Vuokko, V. Vidgren, and J. Londesborough, Yeast. **24**, 741-760 (2007).

104. J. Ratio, K. Kataja, R. Satokari, M. Penttila, H. Soderlund and M. Saloheimo, J. Microbiol. Methods. **65**, 404-416 (2006).

105. Y. Nakao, Y. Kodama, T. Shimonaga, H. Hatanaka, F. Omura, K. Shirahige, S. Furukubo and T. Ashikari, Proceedings of the 31st European Brewing Convention, Venice, 2007, pp. 406-419.

106. T. C. James, S. G. Campbell and U. M. Bond, Proc. IEEE, **90**, 1887-1899 (2002).

107. R. Joubert, P. Brignon, C. Lehmann, C. Monribot, F. Gendre and H. Boucherie, Yeast. **16**, 511-522 (2000).

108. R. Joubert, J. Strub, S. Zugmeyer, D. Kobi, N. Carte, A. Dorsselaer, H. Boucherie and L. Jaquet-Gutfreund, Electrophoresis. **22**, 2969-2982 (2001).

109. J. Brejning, N. Arneborg and L. Jespersen, J. Appl. Microbiol. **98**, 261-271 (2005).

110. D. Kobi, S. Zugmeyer, S. Potier and L. Jaquet-Gutfreund, FEMS Yeast research. **5**, 213-230 (2004).

111. D. Kell, M. Brown, H. Davey, W. Dunn, I. Spasic, and S. Oliver, Nature Rev Microbiol, **3**, 557-565 (2005).

112. Cost efficiencies in the brewing business, web information. Available at: http://www.oceta.on.ca/documents/sleeman_fnl.pdf, accessed April 11, 2008.

113. C. Perry and P. Meaden, J. Inst. Brew. **94**, 64-67 (1988).

114. T. Fujii, H. Yoshimoto and Y. Tamai, J. Ferm. Bioeng. **81**, 538-542 (1996).

115. K. D. Vilanueba, E. Goosens and C. A. Masschelein, J. Am. Soc. Brew. Chem., **48**, 111-114 (1990).

116. M. L. Suihko, K. Blomquist, M. Penttilä, R. Gisler and J. Knowles, J. Biotech. **14**, 285-300 (1990).

117. T. Fujii, K. Kondo, F. Shimizu, H. Sone, J-I. Tanaka, and T. Inoue, Appl. Envir. Microbiol. **56**, 997-1003 (1990).

118. S. Yamano, K. Tomizuka, H. Sone, M. Imura, T. Takeauchi, J. Tanaka and T. Inoue, J. Biotech. **39**, 21-26 (1995).

119. M.E. Penttilä, M.L. Suihko, U. Lehtinen, M. Nikkola, and J.K.C. Knowles, Curr. Genet. **12**, 413-430, (1987).

3

Physiological characterization of brewer´s yeast in high gravity beer fermentations with glucose or maltose syrups as adjuncts

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Abstract

High gravity brewing, which can decrease production costs by increasing brewery yields, has become an attractive alternative to traditional brewing methods. However, as higher sugar concentration is required, the yeast is exposed to various stresses during fermentation. We evaluated the influence of high gravity brewing on the fermentation performance of the brewer´s yeast under model brewing conditions. The lager brewer´s strain Weihenstephan 34/70 strain was characterized at three different gravities by adding either glucose or maltose syrups to the basic wort. We observed that increased gravity produced a lower specific growth rate, a longer lag phase before initiation of ethanol production, incomplete sugar utilization and an increase in the concentrations of ethyl acetate and isoamyl acetate in the final beer. Increasing the gravity by adding maltose syrup as opposed to glucose syrup resulted in more balanced fermentation performance in terms of higher cell numbers, respectively higher wort fermentability and a more favorable flavour profile of the final beer. Our study underlines the effects of the various stress factors on brewer´s yeast metabolism and the influence of the type of sugar syrups on the fermentation performance and the flavour profile of the final beer.

Introduction

When higher productivity and substantial economic savings are the key aspects in process development, high gravity brewing is an attractive approach. It requires the use of wort with higher sugar concentration and because of the higher ethanol concentrations produced, at the end, the beer is diluted to the desired ethanol content. In brewing, the unit of gravity used is degrees Plato. Brewing wort at 12 °Plato (equivalent to 12 g extract per 100 g liquid) ferments to produce beer of 5 % (v/v) ethanol whereas brewing wort of 18 °P produces beer with 7.5 % (v/v) ethanol content. Such beer is diluted further to achieve a final ethanol content of 5 % (v/v) (Blieck et al. 2007). The use of high gravity brewing technology has the advantages of increasing brewery capacity by 20-30 % without additional expenditures for facilities, reducing the cost of energy and labour, and improving the beer smoothness, flavour and haze stability (Stewart 2007a,b). The relatively high ethanol concentrations formed during fermentation promotes increased precipitation of the complex polyphenol-protein material, thus high gravity produced beer has better colloidal stability than standard gravity fermented beer (Bolton and Quain 2006). High gravity brewing also offers a flexibility of the beer type produced as products with different sugar content (light beer) and alcohols levels (low/high alcohol beer) can be produced (Stewart 2007a,b).

The negative effects of high gravity brewing on the brewer´s yeast performance are increased osmotic pressure due to high sugar concentrations at the beginning of the fermentations and elevated ethanol concentrations towards the end of the fermentations. Both of those factors have been implicated as limiting factors for reduced yeast viability and vitality (Pratt et al. 2003). As a result, reduced fermentation rates and incomplete fermentations in wort with more then 18 °P may be observed. Such fermentations result in high residual sugars, mainly maltotriose and maltose, lower ethanol yield and modified flavour profiles and the extent of the osmotic pressure depends on the concentrations of solutes surrounding the medium.

The use of certain adjuncts types to supplement the wort is another cost saving approach used in the high gravity brewing industry. However, unlike wort, adjuncts contain only carbohydrates and not other nutrients, consequently the addition of adjunct to the wort at the beginning of beer fermentation results in modified nutrient balance compared to normal gravity fermentations. Thus, increased initial gravity will result in beer fermentations with reduced cell number and lower

specific growth rate (Casey et al. 1985). The use of sugar syrups as adjuncts reduces also the available amino acid content and the lower specific growth rates lead to lower amino acid uptake rates. Such fermentations are also associated with a modified (abnormal) pattern of sugar uptake and altered levels of production for some of the flavour compounds (Dragone et al. 2007; Boulton and Quain 2006). The modified flavour and aroma compound profile in the final beer produced from the high gravity brewing process is especially pronounced with regards to elevated levels of ethyl acetate and isoamyl acetate, compared to beer produced from lower gravities, but with the same alcohol content.

The objective of the present study was to evaluate the influence of high gravity fermentation conditions, in terms of modified carbon to nitrogen ratio on the brewer´s yeast physiology and fermentation characteristics. Furthermore, the addition of glucose versus maltose syrup adjuncts in high gravity beer fermentations and their influence on brewer´s yeast physiology and the flavour compound formation in the final beer was investigated.

Materials and methods

Wort

All-malt wort with a gravity of 14 °P and pH=5.2 (purchased from Alectia A/S, Denmark), was used for all fermentations. The wort contained 90% carbohydrates of which the fermentable carbohydrates consisted of 4.4 % fructose, 12.5 % glucose, 66.5 % maltose and 16.7 % maltotriose (w/v). The wort also contained non-fermentable carbon sources such as dextrins and β-glucan. $ZnSO_4.7H_2O$, was added to a concentration of 0.1 ppm Zn. For adjusting the wort to higher gravities - 21° and 24° Plato, respectively, highly fermentable syrups - Clearsweet® 95% Refined Liquid Dextrose Corn Syrup (95.5 % glucose, 2,5 % maltose, 1 % maltotriose, 1 % higher saccharides, present in % dry basis (w/w)) and Satin Sweet® 65 % High Maltose Corn Syrup (70 % maltose, 18 % maltotriose, 2 % glucose, 9 % higher saccharides, present in % dry basis) were used as adjuncts. Both syrups were kindly provided by Cargill Nordic A/S. The resulting sugar composition of the different fermentation media used in this study, as measured by HPLC analysis, is summarized in **Table 3-1**. Prior to inoculation, the wort was oxygenated with air until it reached 100 % saturation.

Table 3-1. Sugar composition and free amino nitrogen (FAN) content of the wort at different gravities used in the present study.

Wort ^{o}P	Glucose g l^{-1}	Fructose g l^{-1}	Maltose g l^{-1}	Maltotriose g l^{-1}	Total sugars g l^{-1}	FAN mg l^{-1}
14	12.5	4.4	67.1	16.9	100.9	240
21Gl	98.8	5.2	78.6	18.7	201	210
21M	14.5	4.7	135	35.7	190	220
24Gl	144	5.7	68.2	16.5	235	192
24M	16	5.0	157	42.4	220	201

[a] *Gl represents supplementation with glucose syrup; M represents supplementation with maltose syrup.*

[b] *The numbers 14, 21 and 24, respectively represent the corresponding gravity in the fermentation media.*

Wort density

Wort density was measured using an A. Paar density meter DMA 4500 apparatus (Anton Paar GmbH, Germany) and the gravity was expressed in degree Plato. One degree Plato corresponds to 1 gram of extract per 100 grams of liquid solution, where extract include both fermentable sugars and non fermentable carbon sources such as dextrins and β-glucan (Blieck et al. 2007).

Strain

The flocculent bottom fermenting industrial beer yeast strain Weihenstephan 34/70 (Hefebank Weihenstephan, Freising, Germany) was used in this study. The strain was maintained as a frozen stock culture in 40 % (v/v) glycerol.

Fermentation conditions

For the pre-cultures, the yeast from the stock culture was propagated on YPD plates at 30 °C for four days. A single yeast colony was transferred to 20 ml of 14 °P wort in a sterile 50 ml Falcon

tube and incubated at 25 °C in a rotary shaker at 150 rpm. After 48 hours, the preculture was transferred to a shake flask with 375 ml of fresh wort and incubated for 72 hours.

All fermentations were performed in 2.2 liter bioreactor (Biostat B5; Braun Biotech International, Melsungen, Germany) with a working volume of 1.5 liter. Dissolved oxygen was monitored with an autoclavable polarographic oxygen electrode. The fermentors were integrated with Braun Biotech Multi-Fermenter Control System (MFCS) for data acquisition. Silicone based antifoam agent FD20P in concentration of 0.1 ml/L (Basildon Chemicals, England) with a food gradient quality was used in the fermentations. The reactors were inoculated with a volume of pre-culture, corresponding to 1×10^7 cells/ml. During the cultivation the temperature was maintained at 14 °C and the stirring was set to 90 rpm. Prior to sampling the stirring was increased to 300 rpm for 2 min. The higher stirring allowed better mixing and homogenization of the media and ensured representative sampling. The pH was recorded on-line, but not controlled. After the fermentation was completed, the whole fermentation broth was transferred to a sterile vessel and stored for 14 days at 0°C, for further maturation.

Methylene Blue staining

Viability tests were done using the methylene blue staining method according to EBC Analytica method (Hjortshöj et al. 1992) and the viable yeast cells were counted in a Bűrker-Tűrk cell counting chamber.

Sampling

Samples for analysis of sugars, alcohols and amino acids were collected every 24 hours throughout the fermentation. For measuring the wort density and free amino nitrogen content, samples were collected from the first and the final day of the primary fermentation. For the all of the above analyses, 2-10 ml of fermentation samples were withdrawn from the fermentor, immediately filtered through a Cameo 0.20 µm pore size acetate/glass filters (Sartorius AG, Germany) and stored at -20 °C prior to analysis. Samples for flavour compound analyses were collected after 14 days of maturation, filtered and stored at -20 °C prior to analysis. For determination of the intracellular trehalose content, cell mass samples were collected from the exponential and from the stationary phases of the fermentations. Cells from 5 ml of fermentation broth were harvested by centrifugation, washed 3 times with cold 0.9 % (w/v) NaCl and dissolved in 0.2 M sodium citrate

buffer, pH 4.8. The samples were frozen in liquid nitrogen and stored at -20 °C. Standard deviations between the duplicate fermentation samples were determined to be lower than 5% of the average value for the analysis of sugars, alcohols, amino acids, wort density and flavour compounds determination and lower than 10% of the average value for the analysis of free amino nitrogen content.

HPLC analysis

Carbohydrates and alcohols

A Dionex Summit HPLC system (Synnyvale, CA) was used for analysis of sugars and metabolites from the extracellular medium. All metabolites were detected refractometrically (Waters 410 Differential Refractometer Detector, Millipore Corp., Milford, MA) after separation on an long Aminex HPX-87H column (Biorad, Hercules, CA) at a temperature of 60^{o}C using 5 mM H_2SO_4 as eluent. To allow the separation of the sugars with different degree of polymerization, two Aminex columns were mounted in serial with isocratic elution at 0.40 ml/min. To account for the metabolite concentration both non-diluted and 15 times diluted in eluent samples were analysed. External standards of maltotriose (DP3), maltose (DP2), glucose (DP1), fructose (DP1), glycerol and ethanol were used for external quantification at 6 different levels.

Amino acids

The 20 essential individual amino acids were quantified on Dionex Summit HPLC system after derivatization in alkaline buffer with o-phthalaldehyde (OPA) (primary amino acids) and 9-flourenylmethyl chloroformate (FMOC) and secondary amino acids (Herbert et al. 2001). The amino acid derivatives were separated on a 150 x 4.60 mm, 3 µm, Gemini C_{18} column (Phenomenex, Torrance, CA). Detection was performed using a RF200 fluorescent detector, using 288 nm as excitation wavelength and 305 nm as emission wavelength. Separation was performed using a linear binary gradient 1.0 ml/min of A (20 mM triethylamine adjusted to pH 7.5 using acetic acid) and B (45% acetonitrile, 45% methanol and 10% water). The gradient started with 12% B for 12 min and was then linearly increased to 40% B at 38 min and further increased to 61% for 9 min, after which increased to 100% in 1 min where it was maintained for 2 min before reverting to the starting conditions in 4 min.

Free amino nitrogen (FAN) analysis

The levels of free amino nitrogen (FAN) of the unfermented worts and from the last day of the beer fermentation were determined using the ninhydrin method at 570 nm (EBC 1998). Glycine was used as a standard.

Total protein

For determination of the total protein content, 50 µl of the supernatant was treated with 3 ml Bradfort reagent (Sigma) and the samples were measured on spectrophotometer at 595 nm. Bovine serum albumine was used as a standard at three levels.

Trehalose determination

Prior to extraction, the samples were thawed by rapid boiling for 5 min and the cells were disintegrated using glass beads with size (0.25-0.5 mm) in a Savant Fast Prep FP120 (Savant Industries, N.Y.) (Schulze 1995). After centrifuging, the supernatant was treated with trehalase (0.148 U/ml) (Sigma) in 0.2 M sodium citrate buffer pH 5.7, overnight at 57 ^{o}C with 150 rpm shaking. Liberated glucose was measured in duplicate samples using an enzymatic kit (Unimate 5 Gluc HK, Roche, Switzerland) on an automatic analysis robot (Cobas Mira, Roche, Switzerland) and the samples were normalized with the total protein content. Calibration with glucose as a standard at three different levels was used.

Headspace analysis of esters and higher alcohols

GC-FID analysis of the esters and higher alcohols of the final beer samples was performed using a Perkin-Elmer Autosystem XL gas chromatograph equipped with automatic HS40 XL headspace autosampler. Samples of 5.0 ml were transferred to 20-ml auto sampler vials and capped with butyl-PFTE seals. Samples were thermostated for 30 min at 60°C, pressurized with 3.5 bar helium, and transferred to the GC trough a 0.25 mm ID deactivated fused silica held at 90°C (transfer line). Injection to the analytical column DB-5 (60 m, 0.25 mm, ID 1.0 µm film, J&W Scientific) was done in split mode at a ratio of 1:10 for 1.2 min. The initial oven temperature was set at 35 ºC for 2 min, followed by an increase with 6 ºC/min until the temperature reached 200 ºC. The flame ionization temperature was 250 ºC, and helium was used as the carrier gas at a constant flow of 35

cm/s. Perkin-Elmer Turbochrom Navigator software was used for instrument control. N-butanol was used as an internal standard, and calibrated against all six studied compounds at six levels.

Calculations of specific growth rate, yield coefficients and wort fermentability

The specific growth rate was determined as the slope from the linear function of the natural logarithmic function (ln) of the cell number (cells/ml) and the fermentation time (h) during the exponential growth phase. The yield coefficients were determined as the slope from the linear regression on the corresponding pairs of substrate (total saccharides) and product concentration (glycerol and ethanol).

The percentage fermentability of the wort is the proportion of the wort dissolved solids (extract) which is fermented during the course of the beer fermentation. The percentage fermentability is calculated using the following formula (Briggs et al., 2004):

Fermentability(%) = [(original gravity-final gravity)/(original gravity)]x100

Results

The physiological characterization of the industrial lager beer yeast strain Weihenstephan 34/70 was performed in basic wort at 14 °P and in high gravity wort at 21 °P and 24 °P, achieved with the addition of glucose or maltose rich syrups to the basic wort (for sugar compositions in the different media, see **Table 3-1**).

Effect of the gravity increase and type of sugar syrups on the brewer´s yeast growth

The main products of beer fermentations are ethanol, carbon dioxide, glycerol and yeast biomass. In all of the five studied wort conditions, but especially pronounced for the fermentations at higher gravities, the fermentation profile can be separated into three phases (**Figure 3-1**). During the first lag phase little to no cell growth occurred. Then the cells entered the exponential growth phase, where cell growth, sugar consumption, and product formation take place. After deceleration, the brewer´s yeast cells entered the stationary phase and the cell number remained constant. With the advance of the stationary phase, the cell numbers decreased. Especially pronounced at the higher gravities, in the stationary phase, although there was no active cell growth, the sugar utilization and ethanol and glycerol formation continued at a lower rate than during the exponential growth phase

(**Figure 3-2**). In all cases, the pH profile of the fermentations declined from 5.3-5.1 at the start of the fermentation to 4.2-3.9 (data not shown). With increase in the gravity the sugar syrups slightly acidified the wort, thus the starting pH for high gravity fermentation was slightly lower, but the final pH was not lower than 3.9.

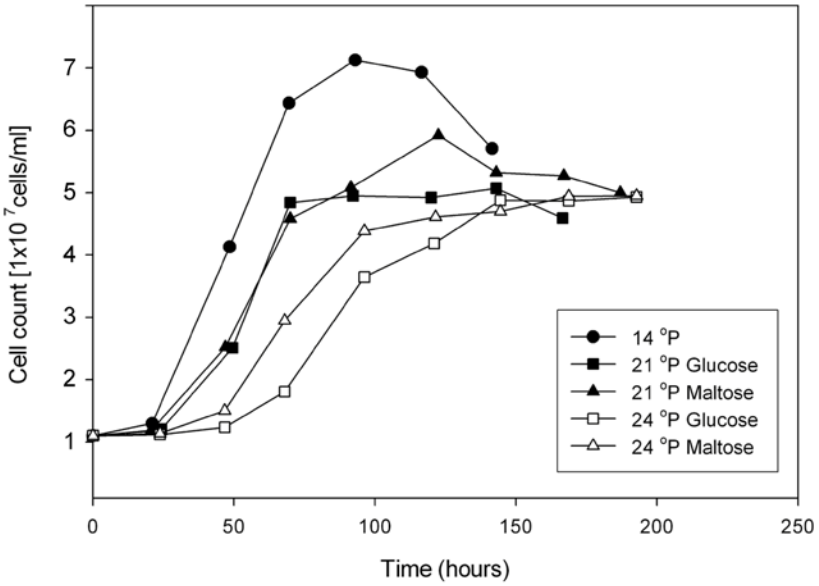

Figure 3-1. Time course of the cell number from the exponential growth phase of the studied fermentations. The number 14, 21 and 24 correspond to the gravity of the wort. Gl and M represents glucose and maltose syrup supplementation, respectively.

With increase in the gravity from 14 °P to 24 °P, both for the glucose and for the maltose supplemented fermentations, both the final cell numbers and the specific growth rate decreased (**Table 3-2**). Among the five studied conditions, the highest specific growth rates and the highest cell numbers in the exponential growth phase were observed for the 14 °Plato fermentations - 0.067 (h^{-1}) and 7.1×10^7 cells/ml, respectively. When maltose syrup was used as a supplement to the wort of 21 °P and 24 °P, higher specific growth rate 0.064 (h^{-1}) and 0.049 (h^{-1}), respectively and higher maximum cell numbers - 4.9×10^7 cells/ml and 4.6×10^7 cells/ml were observed, compared to the glucose supplemented wort of the same gravity - 4.8×10^7 cells/ml and specific growth rate of 0.05 (h^{-1}) for the 21° P fermentations and 4.1×10^7 cells/ml and specific growth rate of 0.044 (h^{-1}) for the 24° P fermentations. With increase in the gravities, also longer time was necessary for the cells to

enter the exponential growth phase and for the initiation of the ethanol production. The yeast cells from 14 °P and 21 °P fermentations entered the exponential growth phase at around 20 and 25 hours of fermentation, respectively. For the 24 °P fermentations, the cells entered exponential growth phase first after 46 hours of fermentation.

Comparison of sugar utilization and alcohol production with increase in the gravity

The sugar uptake in brewer´s yeast fermentations is an ordered, complex and highly regulated process. Glucose, fructose, maltose and maltotriose were the four main fermentable sugars present in the wort (**Table 3-1**). Glucose and fructose were consumed first, followed by maltose. After maltose was consumed to certain extent, then maltotriose started to be utilized. With increase in the gravity, the order of sugar uptake remained the same, but the rate of sugar utilization decreased (**Figure 3-2**). While at 14 °P the fermentations ended with almost complete utilization for all sugars and very small amount of residual sugars left, at the end of the 21 °P and 24 °P fermentations higher amount of residual sugar remained (**Table 3-2**). While for the 21 °P fermentations more complete fermentations in terms of sugar utilization were observed for the maltose supplemented fermentations, for the 24 °P fermentations, both glucose and maltose supplemented fermentations had similar amounts of residual maltose and maltotriose left at the end of the fermentation.

The highest wort fermentability - 80% was achieved for the 21 °P fermentations with maltose syrups supplementation, followed by the 14 °P and 21 °P fermentations with glucose syrups supplementation, both with similar wort fermentability, 76% and 75%, respectively. As expected, 67% wort fermentability was observed for both of the 24° P fermentations with glucose and maltose syrups supplementation. This low fermentability was explained by the high carbohydrate concentrations and low free amino nitrogen concentrations prevailing at these conditions.

The highest ethanol yield based on consumed sugars was observed for the 21 °P fermentations, followed by the 24 °P fermentations. For each of the corresponding gravities, slightly higher ethanol yield was observed for the maltose supplemented fermentations - 0.49 (g/g) and 0.47 (g/g) for the 21 °P and 24 °P, respectively and 0.48 (g/g) and 0.46 (g/g) for the 21 °P and 24 °P glucose supplemented fermentations, respectively. Lowest ethanol yield - 0.45 (g/g) was observed for the 14 °P fermentations. The glycerol yield was found to be higher in the glucose supplemented fermentations - 0.026 (g/g) and 0.027 (g/g) for 21 °P and 24 °P, respectively and lower - 0.018 (g/g) and 0.022 (g/g), for the 21 °P and 24 °P maltose supplemented fermentations, respectively.

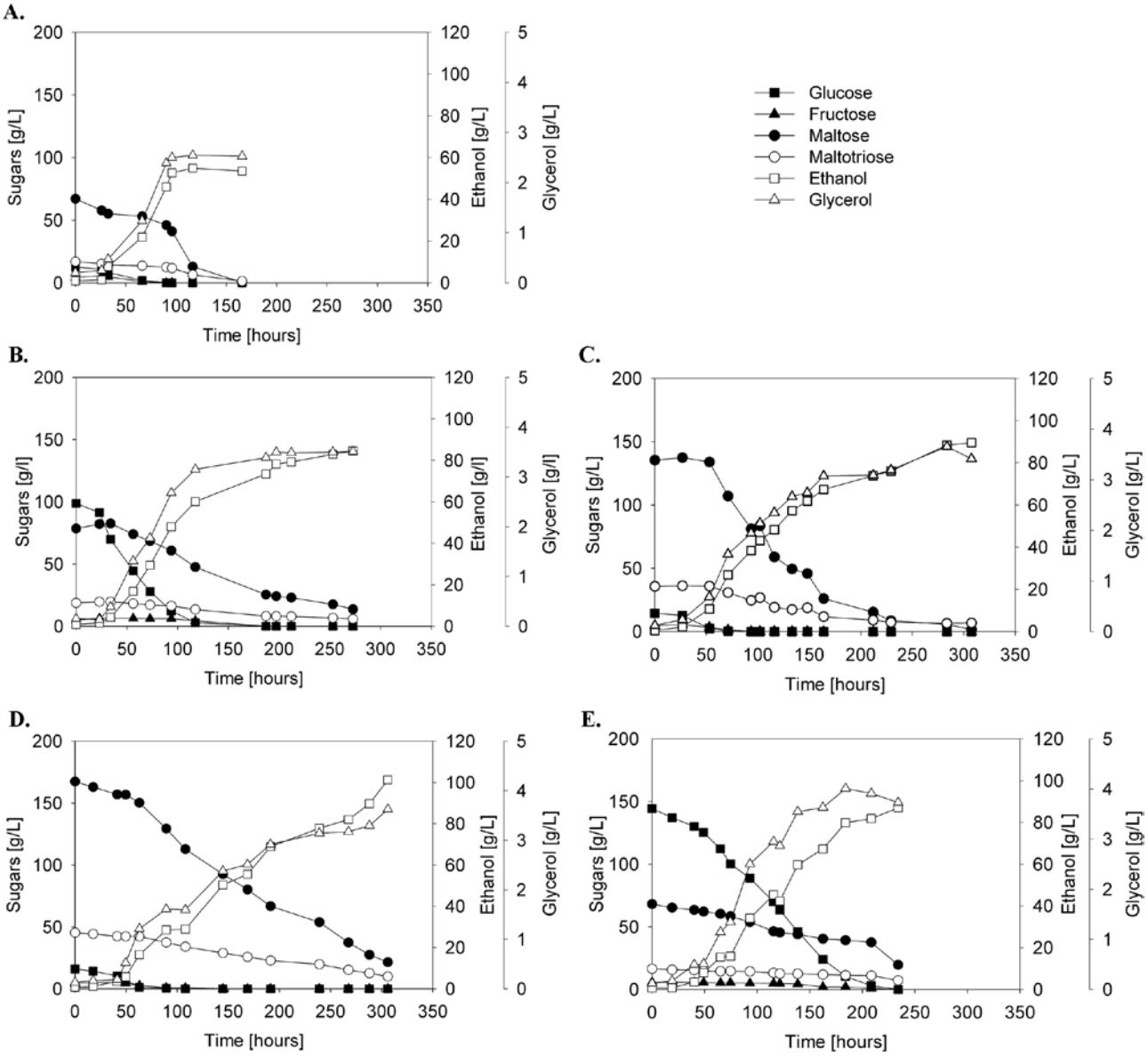

Figure 3-2. Fermentation profiles of the studied fermentations including sugars, ethanol and glycerol concentrations. A. 14 Plato B. 21 P with glucose syrup supplementation, C. 21 P with maltose syrup supplementation, D. 24 P with glucose syrup supplementation, E. 24 P with maltose syrup supplementation. All present values are based on the average of duplicate experiments.

Trehalose accumulation with increase in gravity

To assess the intracellular trehalose accumulation, samples from the early exponential phase and from the stationary phase for each of the fermentations were collected. With increased gravity, the amount of intracellular trehalose increased as well. In the stationary phase, both for the 21 °P and 24 °P fermentations, it was observed that higher intracellular trehalose concentration (**Table 3-2**) was observed for the fermentations with maltose supplementation - 1.98 (g (glucose)/mg (proteins)) and 2.69 (g(glucose)/mg (proteins)), respectively, compared to the glucose supplemented fermentations at 21 °P and 24 °P - 1.82 (g (glucose)/mg (proteins)) and 1.94 g (g (glucose)/mg (proteins)), respectively.

Table 3-2. Growth and wort characteristics of the studied fermentations.

Wort °P	14 °P	21 °P Glucose	21 °P Maltose	24 °P Glucose	24 °P Maltose
Final ^{o} Plato Wort	3.49	5.17	4.29	7.96	7.93
Fermentability (%)	76	75	80	67	67
Final FAN (mg l^{-1})	49	55	52	75	68
Consumed FAN (mg l^{-1})	190	155	148	117	133
% Assimilated FAN	79.6	73.8	76.4	61	66.2
Specific growth rate (h^{-1})	0.067	0.05	0.064	0.044	0.049
Lag phase to ethanol production (h)	21	25	25	40	41
Ethanol yield (g/g)	0.45	0.48	0.49	0.46	0.47
Glycerol yield (g/g)	0.021	0.026	0.018	0.027	0.022
Intracellular trehalose (g glucose) (mg proteins)$^{-1}$ Exponential phase	0.9	1.15	0.97	1.46	1.32
Intracellular trehalose (g glucose) (mg proteins)$^{-1}$ Stationary phase	1.45	1.82	1.98	1.94	2.69

[a] *Values are the average of two independent batch cultivations perform in duplicate (n=2). Standard deviations were determined to be lower then 5% of the average value.*

[b] *Yields are calculated as g of products (ethanol, glycerol) produced per g of total sugars consumed from the exponential phase.*

[C] *The numbers 14, 21 and 24, respectively, represent the corresponding gravity of the wort. Gl and M represents glucose and maltose suryp supplementation, respectively.*

Amino acid consumption

As with the sugar metabolism, the amino acid metabolism in brewer´s yeast is also an ordered process. Previously, the amino acids in beer fermentation have been divided into four groups - A, B, C and D (Pierce 1987) depending on the order of their uptake. Comparison across the 14 °P, 21 °P and 24 °P fermentations showed that the order of amino acid uptake remained the same at all gravities (**Figure 3-3**). The only observed difference was that with increased gravity, related to the decreased specific growth rate, the time for complete amino acids utilization for each of the groups increased. Comparison of amino acid uptake across the different gravities showed that all amino acids from group A were consumed in the first 60 to 70 fermentation hours for the 14 °P and 21 °P fermentations and 90 hours for the 24 °P fermentations. The amino acids from group B were consumed first by the 90-95 hours for the 14 °P and 21 °P fermentations and by the 140 hour point for the 24 °P fermentations. For the amino acids from group C complete uptake was observed after 110-120 fermentation hours for the 14 °P and 21 °P fermentations, while for the 24 °P, complete uptake for the amino acids from group C was observed after 140 fermentation hours. While proline uptake was not observed for the 14 °P fermentations, some proline uptake at a minor rate was observed for the 21 °P and 24 °P fermentations (data not shown). The pattern of the amino acid uptake was very similar between the glucose and maltose syrup supplemented fermentations at the corresponding gravities.

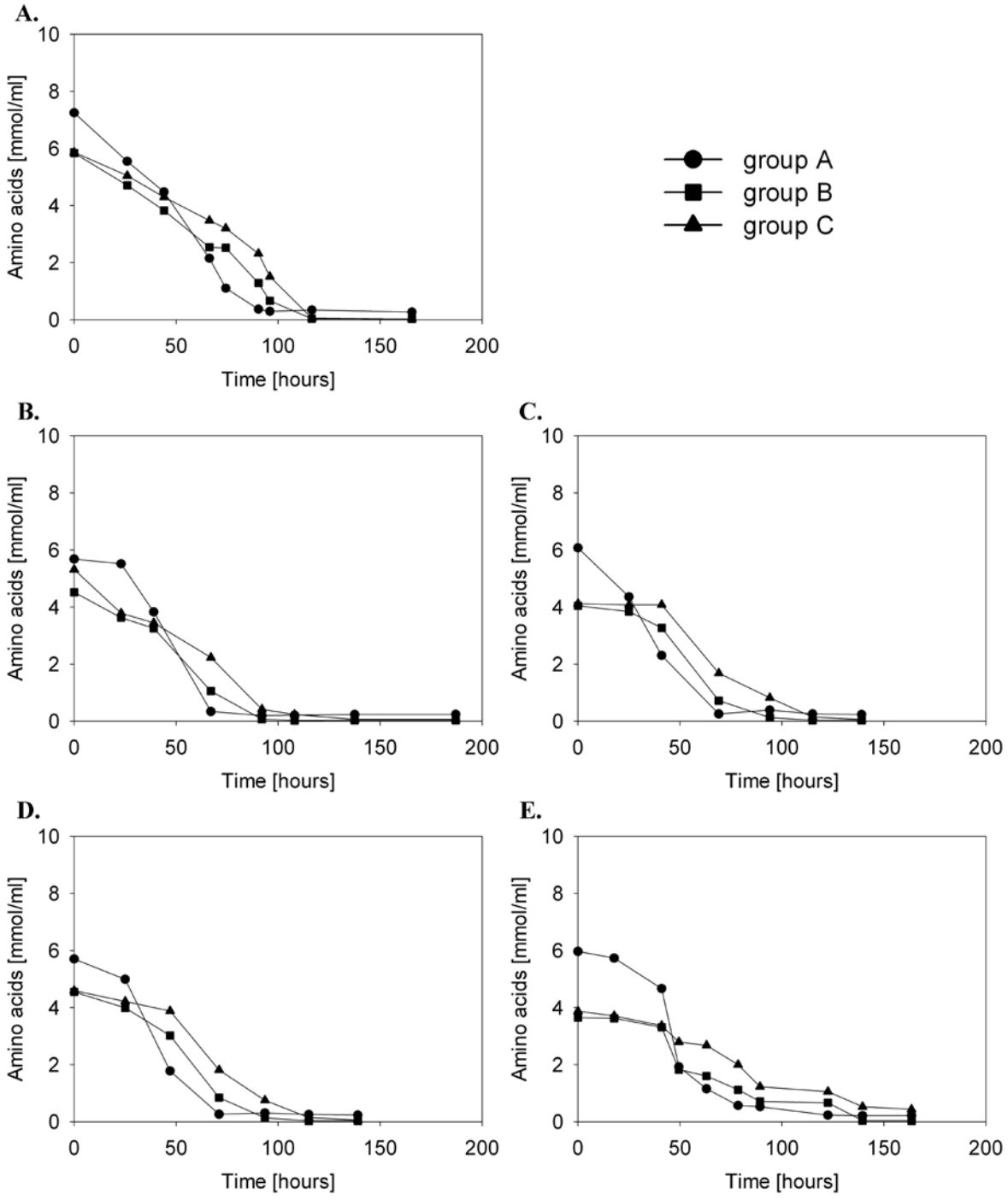

Figure 3-3. Amino acid uptake in the studied fermentations. Group A includes aspartic acid, glutamic acid, asparagine, glutamine, serine, threonine, lysine, arginine. Group B includes valine, methionine, leucine, isoleucine, histidine. Group C includes glycine, phenylalanine, tyrosine, tryptophan, alanine. A. 14 Plato; B. 21 Plato with glucose supplementation; C. 21 Plato with

maltose supplementation; D. 24 Plato with glucose supplementation; E. 24 Plato with maltose supplementation.

Effect of the increased gravity on the free amino nitrogen content

One of the biggest limitations in high gravity beer fermentation is that the use of sugar syrups as adjuncts to increase the gravity dilutes the wort's nitrogen content. Therefore, with the increase in gravity, the levels of free amino nitrogen decrease (**Table 3-2**). In the present study, the initial levels of free amino nitrogen at the beginning of the beer fermentation and the final levels at the end of the beer fermentation were measured. In general, the values for percentage assimilated FAN (% FAN) correlated with the values for percentage fermentability (% fermentability), the specific growth rate and the maximum cell numbers observed. The levels of FAN were highest for the 14 °P fermentations and those fermentations also resulted in the highest amount of consumed FAN (the difference of FAN from the initial and final stage of beer fermentation). Through the course of fermentation around 190 mg l^{-1} of FAN was consumed for the 14 °P fermentations, resulting in 80 % assimilated FAN. The fermentations at 21 °P resulted in consumed FAN in the range of 150-155 mg l^{-1}. Assimilated FAN of 76 % was observed for the 21 °P maltose syrup supplemented fermentations, while the % assimilated FAN was 74 % for the 21 °P glucose syrup supplemented fermentations. The 24 °P fermentations contained the lowest FAN available at the beginning of the fermentation (190-200 mg l^{-1}), and resulted in the highest amount of FAN left at the end of the fermentation (68-72 mg l^{-1}), thus resulting in consumed FAN in the range of 120-130 mg l^{-1}. Again, higher percentage of assimilated nitrogen was observed for the 24 °P fermentations supplemented with maltose syrup - 66 % and lower for the 24 °P fermentations with glucose syrup supplementation - 61 %.

Effect of the increased gravity on the flavour profile of the final beer

To access the flavour profile of the final beer and the influence of increasing gravity on it, the concentration of the esters: ethyl acetate, isoamyl acetate, ethyl hexanoate and ethyl octanoate, higher alcohols - propanol, isobutanol and 3-methyl-butanol and acetaldehyde from the final beer were determined. As high gravity beer fermentations result in final beer with very high alcohol content, in order to compare the concentrations of the flavour compounds at different gravities, the present values of the flavour compounds from the 21 °P and 24 °P fermentations were normalized

to the same ethanol content as of the 14 °P fermentations. With increase in gravity, the biggest difference in the concentrations of the flavour compounds was observed for the ethyl acetate, isoamyl acetate and acetaldehyde. In general, among the esters, with increase in the gravity, the concentrations of ethyl acetate and isoamyl acetate increased from 14.5 mg l^{-1} and 1.1 mg l^{-1}, respectively for the 14 °P fermentations to 35-39 mg l^{-1} and 1.6-2.4 mg l^{-1}, respectively for the 21 °P and 24 °P fermentations. The highest concentrations for both esters at each of the corresponding gravities were observed in the glucose syrup supplemented fermentations. The concentrations of ethyl hexanoate and ethyl octanoate remained similar. With increase in gravity, the acetaldehyde concentrations in the final beer also increased two to four fold. The increase in the acetaldehyde concentrations was also especially pronounced for the glucose supplemented fermentations at the corresponding gravities. With increase in gravity, the concentrations of propanol and isobutanol decreased, while the concentrations of 3-methyl-butanol was slightly higher for the 21 °P fermentations and slightly lower for the 24 °P fermentations, compared to its concentration at 14 °P (**Table 3-3**).

Table 3-3. Concentration of the flavor and aroma components in the final beer. The values for the 21 °P and 24 °P fermentations are reported after data normalization (correction) to the same ethanol content- 5 % (v/v) as 14 °P fermentations.

	Ethyl acetate (mg l^{-1})	**Isoamyl acetate (mg l^{-1})**	**Ethyl hexanoate (mg l^{-1})**	**Ethyl octanoate (mg l^{-1})**	**Acetaldehyde (mg l^{-1})**	**Propanol (mg l^{-1})**	**Isobutanol (mg l^{-1})**	**3-methyl-butanol (mg l^{-1})**
Threshold value	21-30	1.4	0.21	0.9	10	600	100	50-70
14 °P	14.5	1.1	0.10	1.70	1.7	24.5	15.4	55.7
21°P Glucose	37.4	2.5	0.10	0.87	7.2	17.7	14.3	68.5
21°P Maltose	36.0	1.9	0.08	0.70	3.1	16.9	13.8	63.1
24 °P Glucose	39.4	2.1	0.09	0.62	6.3	14.8	9.85	45.5
24 °P Maltose	37.8	1.6	0.05	0.92	5.2	16.9	10.8	47.5

[a] *Values are the average of two independent batch cultivations perform in duplicate (n=2). Standard deviations were determined to be lower than 5% of the average value.*

Discussion

Effect of the increased gravity on the brewer´s yeast sugar metabolism

We observed that by increasing the gravity of the wort there was a lower specific growth rate, a longer lag phase before initiation of ethanol production and incomplete sugar utilization during fermentation. The three main fermentable sugars in brewing fermentation are glucose, maltose, and maltotriose (Dietvorst et al. 2007). Both maltose and maltotriose transport have shown complex kinetics involving high and low affinity transporters and both of those systems are sensitive to glucose-induced inactivation.

Previous research indicated that the genes required for maltose utilisation are repressed by glucose, fructose and sucrose and high levels of maltose and maltotriose remained in the beer when 30% w/v glucose concentrations were used as a high gravity wort adjunct (Stewart et al. 1999). During conditions of limited nitrogen in the wort, which is common in high gravity fermentations, glucose and maltose themselves induce catabolite inactivation of maltose transporters by proteolysis (Rautio and Londesborough 2003).

In the studied fermentations, we observed higher amounts of residual sugars with increase in gravity both for the glucose and maltose supplemented fermentations compared to the normal gravity fermentations (**Table 3-2**). This effect was especially pronounced for the high gravity glucose supplemented fermentations. The glucose repression in *S. cerevisiae* is a complex regulatory system that effects the expression of many genes including those involved in sugar uptake and fermentation (Klein et al. 1998). Thus, the observed high residual maltose concentrations at the end of high gravity fermentations at 21 °P and 24 °P are associated with glucose repression (Stewart et al. 1988) and glucose triggered catabolite inactivation of existing maltose transporter proteins. High ethanol concentration towards the end of the beer fermentation may also play a role in this inactivation, with the known function of ethanol to inhibit the endocytosis and subsequent proteolysis of maltose transporters (Briggs et al. 2004).

In addition, wort fermentability was an important parameter for successful beer fermentation. Besides the obvious economic disadvantages for breweries, in terms of unused substrate and lower final ethanol concentration, incomplete sugar utilization could result in a beer with a higher risk for

contamination by spoilage microorganisms. Especially pronounced at higher gravities, the fermentability dropped from 75-80 % for the 21 °P fermentations to 67 % for the 24 °P fermentations (**Table 3-2**). With increase in the gravity from 21 °P to 24 °P, the effect of the addition of glucose versus maltose syrups diminishes and for both conditions, the fermentations resulted in similar fermentability. Thus, the reduced fermentability at 24 °P is possibly a combination of increased osmotic pressure because of the higher sugar content, reduced FAN content, and increased ethanol toxicity due to the higher ethanol content.

Trehalose accumulation

Trehalose is a non-reducing disaccharide that has been considered to play the role of storage carbohydrate (Lillie and Pringle 1980) and its accumulation is associated with a variety of stress factors such as osmotic stress, temperature shock, high ethanol concentration, low levels of water activity, growth restriction and nitrogen limitation (Briggs et al. 2004). In our fermentations, the intracellular trehalose content increased with increasing gravity. For both the 21 °P and 24 °P fermentations we observed higher intracellular trehalose content for the maltose supplemented fermentations.

Previous research (Majara et al. 1996; Bolton and Quain 2006) has also revealed that the accumulation of trehalose at the late fermentation stages is proportional to the gravity of the wort. Similar findings (Stewart et al. 1999) in high gravity beer fermentations suggested that intracellular trehalose accumulates to higher concentrations in the fermentations with the addition of maltose syrup to the basic wort. For each of the corresponding gravities (Beney et al. 2001), the cells grown in high gravity maltose wort exhibited higher viabilities than the cells grown in high gravity glucose wort.

In glucose rich media, trehalose accumulation occurs during the transition between exponential growth and entry into the stationary phase and when other nutrients are limited or during the onset of diauxie. The higher intracellular trehalose concentrations observed in the stationary phase of the fermentations are a response to the increased stress caused by the combination of the high ethanol concentrations and enhanced nitrogen limitation (Briggs et al. 2004).

We observed that the physiological state of the brewer´s yeast cells is extremely important, especially considering the fact that in the industrial beer production process, the brewer´s yeast is

reinoculated ("repitched") for several fermentation cycles. One of the disadvantages of high gravity beer fermentation is that the extreme conditions imposed on the brewer´s yeast reduces the number of repitching cycles (Bolton and Quain 2006).Therefore intracellular trehalose content is an important parameter of the yeast's physiological state.

Reduced free amino nitrogen and amino acids utilization

The degree of FAN utilization during the course of beer fermentation is one of the indicators for a successful fermentation. However, the use of pure sugar syrups to increase the gravity, dilutes the available free amino nitrogen concentrations as well as other important nutrients in the wort such as biotin, zinc, calcium, magnesium and other microelements and growth factors.

In the present study, the increase in the gravity by the addition of glucose or maltose rich syrups to the media resulted in free amino nitrogen concentrations below the levels necessary for successful fermentation performance. As FAN also partially measures compounds that cannot be utilised by the yeast, typical FAN values from the end of the beer fermentations are in the range of 40-50 mg l^{-1}. Similar values were observed in the present study for the 14 °P and 21 °P fermentations but higher amounts of FAN in the range of 68-75 mg l^{-1} were left at the end of the 24 °P fermentations.

The higher amount of residual FAN left at the end of the fermentations could be correlated with the lower specific growth rate and is possibly an indicator of yeast growth limiting factors other than nitrogen content in the 24 °P fermentations. Maltose supplementation in high gravity beer fermentation at 20 °P using dry yeast of Weihenstephan 34/70 has been reported to result in a higher percentage of assimilable FAN - 76%, compared to the glucose supplemented fermentations - 63.5% (De Rouck et al. 2007). Our results, using fresh inoculums of Weihenstephan 34/70 in the 21° P fermentations, showed a similar trend, though the difference in fermentability was not as pronounced. However, at the 24 °P fermentations, there was no significant difference between the percentage of assimilable nitrogen for the glucose and maltose syrup supplemented fermentations. Thus, the effect of more pronounced nitrogen limitation and increased ethanol concentrations at 24 °P fermentations diminishes the effect of the type of the carbon source in the studied very high gravity fermentations.

The yeast growth and the fermentation profile are influenced by both limiting and excessive presence of assimilable nitrogen. The usable FAN in the wort is directly associated with the formation of flavours, such as the production of esters and higher alcohols and is important for a

production of beer with good organoleptic characteristics. There is a risk that diluted free amino nitrogen concentrations result in beer with elevated levels of higher alcohols (Boulton and Quain 2006). On the other hand, high levels of FAN in the finished beer of the 24 °P fermentations could also result in higher concentrations of aldehydes such as acetaldehyde, 2-methylpropanal, 2-methylbutanal and 3-methylbutanal and contribute to the staling flavour of the aged beer (De Rouck et al. 2007).

Amino acid uptake of the brewer´s yeast

Free amino acids affect the cell mass accumulation, the yeast vitality and ethanol production (Lekkas et al. 2007). The amino acids present in wort are divided into four classes based on their order of assimilation from the wort during the course of beer fermentation. The amino acids from the first two groups A and B are required for anabolic processes (for example protein synthesis) and they are taken up by permeases that are not affected by the nitrogen catabolite repression. It is known that during the uptake of amino acids of group C, nitrogen catabolite repression is active (Briggs et al. 2004). Our results also confirm that the amino acids from group C are taken up after the amino acids from group A have been consumed (Fig. 3). Proline is the only amino acid present in group D and it is the least preferred amino acid by brewer´s yeast. Proline normally is not utilized during beer fermentation since its oxidation requires mitochondrial oxidase (Wang and Brandriss 1987) and as beer fermentation is an anaerobic process, the synthesis of this enzyme is repressed. However, with increase in the gravity from 14 °P towards 24 °P, we observed some proline uptake (data not shown) for the 21 °P and 24 °P fermentations. Under our experimental conditions, the fermentations were not strictly anaerobic but rather microaerobic, thus the observed partial proline uptake is an indicator of pronounced limitation of assimilable nitrogen sources present in the wort, as the cells attempt consumption of the least preferred nitrogen source. This observation points to the need for further elaboration of the role of proline in brewer´s yeast metabolism.

Flavours

The most abundant esters and higher alcohols in beer are ethyl acetate and isoamyl acetate, the amyl alcohols and isobutanol (Younis and Stewart 1998). In this study, with increase in gravity, the concentration of the higher alcohols- propanol, isobutanol and 3-methyl-butanol, slightly decreased. But there were no significant differences in the concentrations of glucose and maltose supplemented fermentations at the corresponding gravities.

Higher alcohols are the precursors of the most flavour active esters and their synthesis is linked to protein production. The main root for the ester formation is catalysed by alcohol acyltransferase, with an alcohol and an activated fatty-acyl CoA molecule as substrates (Briggs et al. 2004).

In our fermentations, with increased gravity, the most significant changes in the flavour compounds from the final beer diluted to the same alcohol content- 5 % (v/v) were observed in increased concentrations of ethyl acetate, isoamyl acetate and acetaldehyde. Ethyl acetate contributes with fruity flavour and isoamyl acetate contributes with banana flavour. However, when present in excessive concentrations, their flavour influence is most often negatively accepted by the consumer. They are normally found in beer produced from average gravity- 10° -12 °P in concentrations below their threshold values of 30 mg l^{-1} and 2 mg l^{-1}, respectively (Casey et al. 1985). In this study, the ethyl acetate and isoamyl acetate concentrations of the final beer from the 14 °P fermentations were in the concentration range normally found in commercial lager beer. However, by increasing the gravity from 14 °P to 24 °P, the concentration of ethyl acetate and isoamyl acetate in the final beer, increased more than two fold. It has previously been shown that high gravity brewing (>16°Plato) is associated with disproportionate higher levels of esters, particularly ethyl acetate and isoamyl acetate (Stewart 2007b).

(Saerens et al. 2008) have also found that the acetate ester levels in fermentation with synthetic medium using maltose as the carbon source and the lager beer yeast strain CMBS SS01, increased with 50 % when the carbon content of the fermentation medium was increased from 8 % maltose to 20 % maltose. Both for the 21 °P and 24 °P fermentations in this study, the ethyl acetate and isoamyl acetate concentration of the final beer were slightly lower for the maltose supplemented fermentations compared to that of the glucose supplemented fermentations. Previous work (Younis and Stewart 1998) using synthetic media suggested that fermentations of very high gravity maltose predominant wort, results in lower levels of these volatiles compared to fermentations with glucose and fructose predominant wort, with similar FAN levels.

There are a number of reasons which have been attributed to the cause of lower levels of esters from the maltose syrup supplemented fermentations. One of those is maltose inhibition on the transport of the volatile compounds out of the cell by altering the plasma membrane (Younis and Stewart 1998), another possibility is that as a result of the maltose metabolism, lower levels of acetyl-CoA have been produced, thus resulting in lower esters concentrations as a result of lack of substrate (Shindo et al. 1992).

Acetaldehyde formation occurs in the exponential growth phase but later on, during the stationary phase, its production usually declines. Acetaldehyde is an important intermediate of the glycolysis and during formation of ethanol. Thus, as expected, higher acetaldehyde concentrations were observed for the high gravity fermentations than normal gravity fermentation. Acetaldehyde´s flavour threshold value is in the range of 10-20 ppm and its concentration normally found in commercial lager beer is either below or in the range of its threshold value. In some cases acetaldehyde can persist in beer above that value resulting in an unpleasant "grassy" flavour (Boulton and Quain 2006). The use of adjuncts with high glucose concentrations inhibits the fermentation rate and yeast growth and a disproportionate increase in the formation of acetaldehyde has been observed (Briggs et al. 2004). Even though that higher acetaldehyde concentrations were observed in the glucose supplemented fermentations, for all of the studied fermentations in the present study, the acetaldehyde concentrations remained within the desired concentration range.

During the course of high gravity beer fermentation, brewer's yeast is exposed to a number of stressful conditions such as high osmotic pressure caused by the high glucose concentrations at the beginning of the fermentations and ethanol stress imposed by the elevated ethanol concentrations towards the end of the fermentation. When sugar syrups are used as adjuncts to achieve higher gravity, the obtained wort contains lower than the minimum required free amino nitrogen concentration for achieving efficient brewer´s yeast fermentation. Thus, brewer´s yeast is exposed to an additional stress, caused by the induced nitrogen limitation and possible limitation of other nutrients, resulting in restricted growth. Our study underlines the effect of the various stress factors on the brewer´s yeast metabolism and the influence of the type of sugar syrups on the fermentation performance and the flavour profile of the final beer. The combination of the above mentioned factors led to poorer yeast growth, longer fermentation times and incomplete fermentation with higher amounts of residual sugars at the end of the fermentations. Additionally, the flavour profile of the final beer was adversely affected by the high concentrations of ethyl acetate and isoamyl acetate. In order to minimize the effect of the negative fermentation performance, the choice of the type of sugar syrups, used to increase the gravity, is of important consideration. The use of maltose syrup to increase the gravity, assuming that there is no additional FAN supplementation, resulted in a more balanced fermentation performance in the 21 °P fermentations in terms of higher cell numbers, a higher specific growth rate, respectively higher wort fermentability and a more favored

flavour profile of the final beer in terms of lower ethyl acetate, isoamyl acetate and acetaldehyde concentrations.

The unusually high amount of residual FAN at the end of the 24 °P fermentations also revealed that there are other growth limiting factors in the beer fermentations when sugar syrups are used to achieve higher gravities. Thus, further investigation is needed to unravel the mechanisms behind the effect of stuck fermentations and the complexity of the metabolic yeast response to both nitrogen and other growth factor limitations and glucose repression.

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References

Beney L, Marechal P, Gervais P (2001) Coupling effects of osmotic pressure and temperature on the viability of *Saccharomyces cerevisiae.* App Microbiol Biotech 56:513-516

Blieck L, Toye G, Dumortier F, Verstrepen K, Delvaux F, Thevelein J, Van Dijck P (2007) Isolation and characterization of brewer´s yeast variants with improved fermentation performance under high-gravity conditions. App Environ Microbiol 73:815-824

Boulton C, Quain D (2006) Brewing Yeast and Fermentation, Blackwell Science Ltd, Oxford

Briggs D, Boulton C, Brookes P, Stevens R (2004) Brewing Science and Practice, Woodhead Publishing Ltd, Cambridge

Casey G, Chen E, Ingledew W (1985) High gravity brewing: production of high levels of ethanol without excessive concentrations of esters and fusel alcohols. J Am Soc Brew Chem 43:179-182

De Rouck G, De Clippeleer J, Poiz S, De Cock J, van Waesberghe J, De Cooman L, Aerts G (2007)

Prolonged flavour stability by production of beer with low residual FAN using active dry yeast. Proc 31st Congr Eur Brew Conv,Venice

Dietvorst J, Blieck L, Brandt R, Van Dijck P, Steensma H (2007) Attachment of MAL32- encoded maltase on the outside of yeast cells improved matotriose utilisation. Yeast 24:27-32

Dragone G, Mussatto S, Almeida e Silva J (2007) High gravity brewing by continuous process using immobilized yeast: Effect of wort original gravity on fermentation performance. J Inst Brew 113: 391-398

European Brewery Convention (1998) Analytica-EBC. Fachverlag Hans Carl, Nürnberg

Herbert P, Santos L, Alves A (2001) Simultaneous quantification of primary, secondary amino acids, and biogenic amines in musts and wines using OPA/3-MPA/FMOC-Cl fluorescent derivatives. J Food Sci 66:1319-1325

European Brewery Convention (1992) EBC Analytica Microbiologica. Fachverlag Hans Carl, Nürnberg

Klein C, Olsson L, Nielsen J (1998) Glucose control in *Saccharomyces cerevisiae*: the role of MIG1 in metabolic functions. Microbiology 144:13-24

Lekkas C, Stewart G, Hill A, Taidi B, Hodgson J (2007) Elucidation of the role of nitrogenous wort components in yeast fermentation. J Inst Brew 113:3-8

Lillie S, Pringle J (1980) Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: responses to nutrient limitation. J Bacteriol 143:1384-94

Majara M, O´Connor-Cox E, Axcell B (1996) Trehalose- an osmoprotectant and stress indicator compound in high and very high gravity brewing. J Am Soc Brew Chem 54:149-154

Pierce J (1987) Horace Brown Memorial Lecture: The role of nitrogen in brewing. J Inst Brew 93:378–381

Pratt P, Bryce J, Sweart G (2003) The effect of osmotic pressure and ethanol on yeast viability and morphology. J Inst Brew 109:213-228

Rautio J, Londesborough J (2003) Maltose transport by brewer´s yeasts in brewer´s wort. J Inst Brew 109:251-261

Saerens S, Delvaux F, Verstrepen K, Van Dijck P, Thevelein J, Delvaux F (2008) Parameters affecting ethyl ester production by Saccharomyces serevisiae during fermentation. App Environ Microbiol 74:454-461

Schulze U, Larsen M, Villadsen J (1995) Determination of intracellular trehalose and glycogen in *Saccharomyces serevisiae*. Anal Biochem 228:143-145

Shindo S, Murakani J, Koshino S (1992) Control of acetate ester formation during alcohol fermentation with immobilized yeast. J Ferm Bioeng 73:370-374

Stewart G (1999) High gravity brewing. Brew Guard 128:31-37

Stewart G (2007a) High gravity brewing- the pros and cons. New Food 1:42-46

Stewart G (2007b) The influence of high gravity wort on the stress characteristics of brewer´s yeast and related strains. Cerevisia 32:37-48

Stewart G, D´Amore T, Panchal C, Russel I (1988) Factors that influence the ethanol tolerance of brewer´s yeast strains during high gravity wort fermentations. Tech Q- Master Brew Assoc Am 25:47-53

Wang S, Brandriss M (1987) Proline utilization in Saccharomyces cerevisiae: sequence, regulation, and mitochondrial localization of the PUT1 gene product. Mol Cell Biol 7:4431-4440

Younis O, Stewart G (1998) Sugar uptake and subsequent ester and higher alcohol production by *Saccharomyces cerevisiae*. J Inst Brew 104:255-264

4

Assessing the chromosomal stability of the brewer´s yeast Weihenstephan 34/70

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The content of this chapter is included in short communication in preparation for publication

Key words: Brewer´s yeast, karyptotyping, chromosomal rearrangements

Abstract

Lager beer yeast strains pose unique characteristics that distinguish them from the yeast *Saccharomyces cerevisiae*. They belong to the group of *Saccharomyces pastorianus* and are considered a polyploid species hybrid of *S. cerevisiae* and other closely related *Saccharomyces* species. In the present study, the lager beer strain Weihenstephan 34/70, was streaked out from a single stock and observed during ten consecutive re-platings. Samples from plating 1, 5 and 10 were examined by pulsed field gel electrophoresis (PFGE). For two of the studied colonies, the karyotyping showed shifts in chromosomal bands by the 5^{th} and the 10^{th} generation, respectively, indicative of translocation or deletion events.

Introduction

Lager beer yeast strains posses unique characteristics that distinguish them from other yeasts. A number of studies examining essential for the beer fermentation genes have shown that in almost all cases, two classes of essential genes are present in the genome. One class with genes that closely resembles genes from *S. cerevisiae* and another class with more divergent patterns, with similarity to *S. bayanus* (Rainieri et al., 2006). The most obvious difference between the sequenced *S. cerevisiae* (Goffeau et al., 1996) and the lager brewing yeast strains is their ploidity number. Originally assigned to the taxon of *Saccharomyces cerevisiae*, today it is accepted that lager beer yeast strains belong to the group of *S. pastorianus* and they are considered to be polyploid species hybrids of *S. cerevisiae* and other closely related *Saccharomyces* species (Smart et al., 2007). Both *S. cerevisiae* and *S. bayanus* are members of the *Saccharomyces* sensu stricto group. They show high conservation of synteny and can be well aligned at nucleotide levels. Ribosomal DNA sequence analysis suggests divergence times of 5-20 million years between them. The average nucleotide percent identity between the two species is reported to be 80% in the coding regions and 62% in the non coding regions (Kellis et al., 2003). A BLASTn analysis for some lager-strain specific genes has demonstrated approximately 85-98% homology to those of *S. cerevisiae* (Bond et al., 2004).

Such brewing strains most often have three or more copies of each chromosome. In addition, while the sequenced *S. cerevisiae* strain has one copy of the genes responsible for maltose fermentation, lager brewing yeasts contain ten or more sets of *MAL* genes (Dietvorst et al., 2007). Furthermore, brewing yeast strains contain specific genes which are not found in baker's yeast, as for example Lg-AFT1, a gene encoding an alcohol acetyltransferase, is present only in the lager brewer´s yeasts (Yoshimoto et al, 1998).

With advances in DNA sequencing technologies in recent years, the possibility of whole genome sequencing of the first lager beer yeast strain, the popular Weihenstephan 34/70, became more feasible. First reported by Nakao *et* al. in 2003, a combination of shotgun sequencing and cosmid library sequencing was used to achieve 348 000 total sequence reads of its genome, corresponding to a 6.5-fold coverage. The minimum size of lager brewing yeast genome has been determined to be 23.2 Mb which is almost twice the size of the *S. cerevisiae* genome (Kodama et al., 2005).

Mapping contigs of the lager brewing yeast genome to those of *S. cerevisiae* also revealed three kinds of chromosomes- Sc-type (*Saccharomyces cerevisiae*-, homologous type), non-Sc- (non *Saccharomyces cerevisiae*-, homoeologous type) and various mosaic types (Nakao *et* al. in 2003) (**Figure 4-1**).

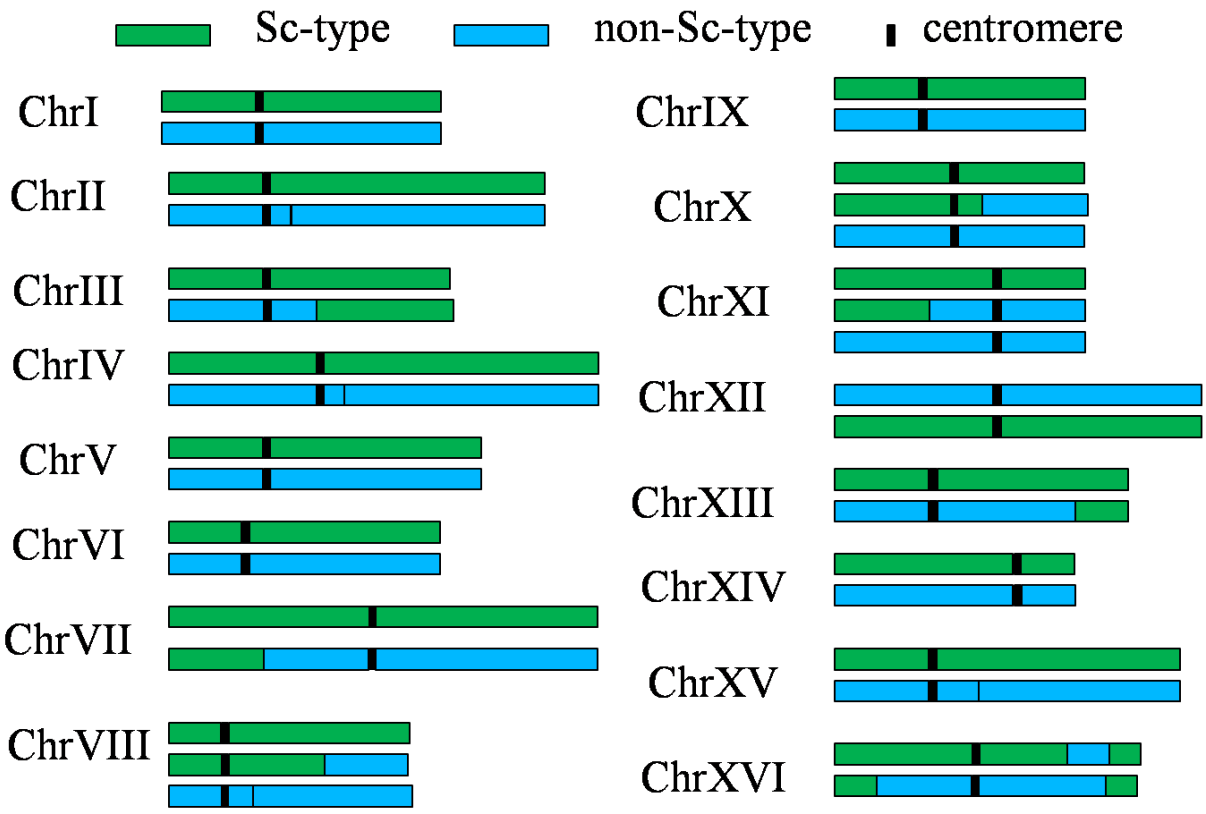

Figure 4-1. Chromosomal structure of the lager brewing yeast strain Weihenstephan 34/70 (adapted from Kodama et al., 2005). Three types of chromosomes are observed: Sc-type (*Saccharomyces cerevisiae*-, homologous type), non-Sc- (non *Saccharomyces cerevisiae*-, homologous type) and various mosaic types.

In the mosaic type, some of the chromosomal breakpoints appeared within ORFs, thus creating hybrid ORFs. The lager brewing yeast genome contains at least eight mosaic chromosomes and on chromosomes VIII, X and XI all three chromosome types can be found (**Figure 4-1**). More recombination break points are usually expected close to the telomere regions as these are subject to more frequent rearrangements (Kodama et al., 2005; Smart, 2007). A number of studies to determine the mosaic structure of the lager beer yeast chromosomes, using Southern Blot hybridization, restriction fragment length polymorphism (RFLP) and amplified fragment length

polymorphism (AFLP) (Hansen and Kielland-Brandt, 2003; Smart, 2007) have been performed. The Southern hybridisation experiments have given further evidence that the gene types with low similarity to *S. cerevisiae* genome show high similarity to *S. bayanus* genes (Tamai et al., 1998).

Determination of the ploidity, the types of chromosomes and the number of copies they exist in is of essential importance for revealing the complex genome of the lager brewing yeast. Southern hybridisation, sequencing and single-chromosome transfer have been used in the investigation of the chromosome structure of the lager beer yeast. These analyses have shown that *S. pastorianus* has a very complex chromosome structure, where chromosomes or chromosome parts can be up to tetraploid (Kodama et al., 2005). In general, *S. pastorianus* is regarded as an allotetraploid, but aneuploidity up to pentaploidity can be the case for specific genes. Most probably, the unique genome structure of the lager brewing yeast strains has been maintained because of the poor mating phenotype associated with aneuploidity (Codon et al., 1998). The phenomenon of alloploidity is seen among most industrial strains and it is hypothesized to be mechanism for promoting evolutionary change by adapting to new environments. Chromosomal changes of industrial yeast populations have by some researchers been explained to be the yeasts way to respond to industrial conditions that can be very harsh.

In recent years, the increased use of DNA fingerprinting techniques has shown evidence that the brewing yeast genome can and does change through chromosomal rearrangements (Meaden 1990; Boulton and Quain, 2006) by exchanging, adding or losing of DNA, resulting in altered chromosome size or even in complete loss of a chromosome. The karyoptyping techniques have provided a tool, by which chromosomes are separated and characterized by size, thus possible chromosomal rearrangements can be observed (Smart 2007). Karyotyping studies have shown changes in chromosome size (Sato et al., 1994; Casey, 1996) and RFLP fingerprinting (Meaden 1990) has been used to associate the genetic changes with the process performance of a variant strain.

In order to asses the chromosomal stability of lager beer yeast strains, in the present study karyotypes of the popular lager beer yeast strain Weihenstephan 34/70, originating from the same stock were followed for ten consecutive re-inoculation cycles.

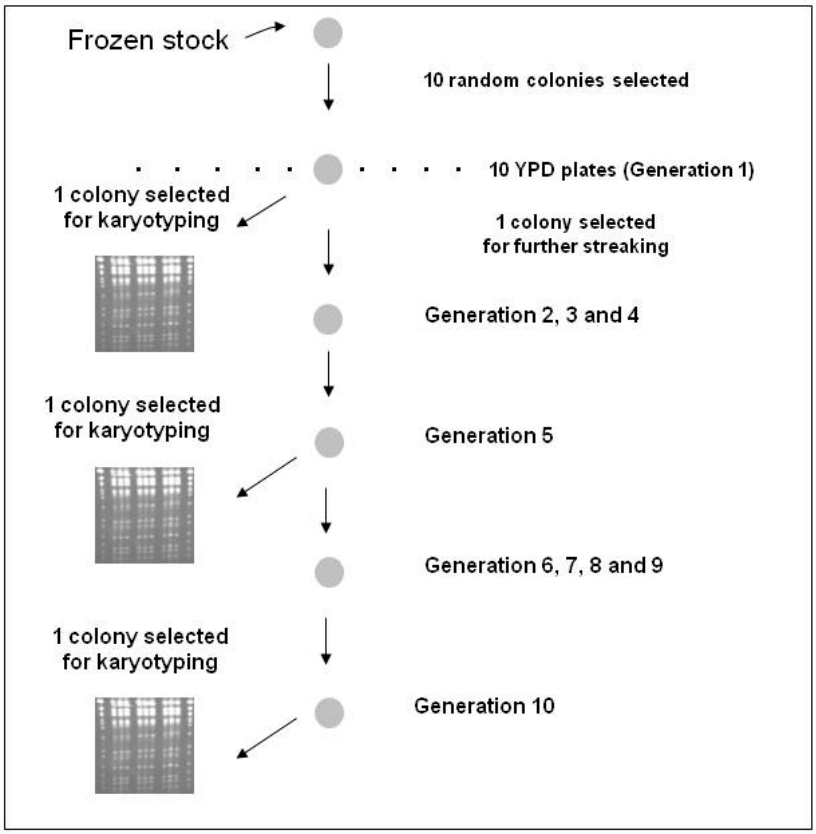

Figure 4-2. Overview of the experimental design for studying the genome stability of strain Weihenstephan 34/70 in ten consecutive reinoculation cycles (here in called generations). *In the present study, each generation represents one re-inoculation cycle.*

Materials and methods

Experimental design

Starting from 40 % (v/v) glycerol frozen stock of Weihenstephan 34/70, a YPD plate was streaked out and incubated at 30 ºC for 2 days. From the plate, 10 colonies of average size were selected for further analysis. Each colony was streaked out on a separate YPD plate. Thus, 10 YPD plates represented re-inoculation cycle 1. After two to three days of incubation, one colony from each of these 10 plates was again selected for further streaking. This procedure was repeated continuously until re-inoculation cycle 10 was obtained (**Figure 4-2**).

In order to assess the chromosomal stability of strain Weihenstephan 34/70 for ten consecutive re-inoculation cycles, the karyotypes of generation 1, 5 and 10 were examined by pulsed field gel electrophoresis (PFGE). For simplicity, in the present study, the re-inoculation cycles are here in referred to as generations. One colony from each of the 10 plates of the respective generation was used to make a preculture for the yeast chromosome plugs.

Preculture and CHEF Genomic DNA Plugs production

The precultures for the strain Weihenstephan 34/70 were made by inoculating one colony in an autoclaved test tube containing 15 ml 14 ˚P wort. The precultures were incubated at 25˚C until an OD_{600} of around 2 was reached.

CHEF Genomic DNA Plug Kit (Bio-Rad) was used for the preparation of 1 % agarose genomic plugs according to the manufacturer instructions (Bio-Rad manual). In order to improve the band intensity during PFGE electrophoresis and to determine the optimal cell number per plug, several optimisation experiments were performed. The optimal determined cell number per plug for strain Weihenstephan 34/70 was 8.0×10^7 cells/plug. The produced plugs were kept in falcon tubes containing wash buffer from the CHEF BioRad kit and placed at 4 ˚C.

Pulsed-Field Gel Electrophoresis

Pulsed-Field Gel Electrophoresis (PFGE) allows the separation of DNA ranging in size from few Kb pairs to ten Mb pairs. For performing the PFGE gel electrophoresis, contour-clamped homogenous electric field equipment, CHEF-DR™ II and CHEF- mapper (Bio-Rad Laboratories), were used. Several different electrophoresis programs were tested in order to achieve good separation of the chromosomes on the gel and a two step program of 46 hours was selected. The first step in the program was set for 38 hours with switching time of 75-90 sec at 4.5 V/cm, followed by a second step of 8 hours with switching time of 90-120 sec at 6 V/cm. In both steps the switching angle was set to +/- 60° and the ramp was linear.

Prior to electrophoresis, thorough washing of the CHEF apparatus with sterile deionised water and 1% NaOH was done and the CHEF apparatus was cooled to 14 ˚C. The 1% CHEF quality agarose gel was prepared according to the manufacturer instructions (Bio-Rad manual, 2007). As running buffer, 0.5 x TBE buffer was used. After completion of the CHEF gel electrophoresis program, the gel was stained in 0.5 µg/ml ethidium bromide for at least 30 minutes using cold 0.5xTBE buffer. The gel was then distained for 30 minutes in 0.5xTBE buffer. Both staining and distaining were

performed under gentle shaking. A commercial preparation of *S. cerevisiae* strain YNN295 (Bio-Rad) was used as a standard to estimate size of the bands. The PFGE gels were run in duplicates and good reproducibility between the same batches of plugs in the different runs was obtained.

Results and discussion

Initially, to assess the similarity between the genome of the Weihenstephan 34/70 strain and its suggested ancestors, *S. cerevisiae* and *S. bayanus*, genomic plugs of Weihenstephan 34/70 and the two wildtype *Saccharomyces* yeasts were prepared and the karyotypes of the three strains analysed on a single PFGE gel. The comparative karyotyping of the three strains showed a distinctive chromosomal pattern for each of the strains (data not shown). However, the lager brewer's yeast clearly had chromosomal band patterns in common with each of the two others. Thus, the observed karyptopying patterns are in agreement with the hypothesis that *S. pastorianus*, the species which strain Weihenstephan 34/70 belongs (Kodama et al., 2005), is likely a hybrid of *S. cerevisiae* and *S. bayanus*.

In order to assess the genomic stability of the lager brewer´s strain Weihenstephan 34/70, ten selected colonies, originating from a frozen stock of strain Weihenstephan 34/70 were streaked out on YPD plates for 10 consecutive re-inoculation cycles. A single, average sized colony from generation 1, 5 and 10 of the re-inoculation cycles were selected and subsequent genomic plugs were prepared and further analysed using PFGE gel electrophoresis.

Comparative karyotyping analysis from generation 1, 5 and 10 for strain Weihenstephan 34/70

For most of the chromosomes, no significant variation in the karyotypes were observed between the different generations originated from the same single colony and the pattern of the chromosome bands and the overall intensity of the bands seemed to be conserved throughout the generations of the same colony (**Figure 4-2**). There were only slight variations in intensities for some of the chromosomes of the different colonies from the same generation. The differences were especially pronounced for the chromosomes with bigger size, at around 945- 1100 kb and around 1600-2200 kb, where the intensities differed from colony to colony (data not shown). The stronger intensity is

possibly an indication that there are more copies present of the chromosomes with that particular size.

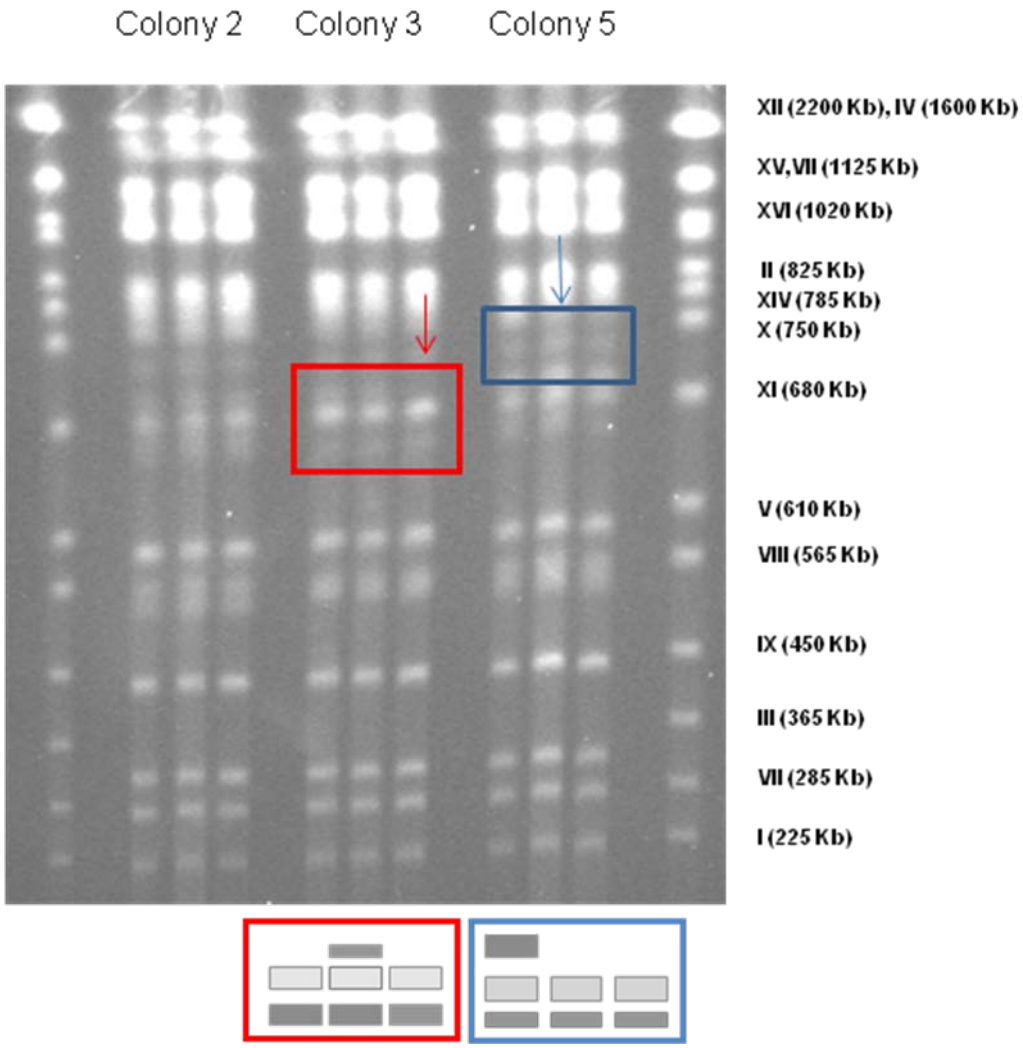

Figure 4-2. PFGE gel electrophoresis for colonies 2,3 and 5 followed for ten consecutive re-inoculation cycles. *The marker used is a commercial marker presenting the chromosomes of the haploid S. cerevisiae strain, YNN295. The chromosome names and sizes for the marker are shown on the right. The arrows indicates the observed missing bands with approximate size of 680 Kb from generation 10 for colony number 3 (red arrow) and 690 Kb from generation 5 for colony number 5 (blue arrow). Schematic representations of the observed translocations are present at the bottom of the figure.*

However, for colony number 5, a chromosome band of approximately 760 Kb, slightly bigger than the corresponding band of 750 Kb for chromosome X from *S. cerevisiae* has been translocated or deleted in the 5th generation. As this band is present in the first generation, this band rearrangement must have taken place between the 1st and the 5th generation. Similarly, for colony number 3, a

chromosome band of approximately 690 Kb, just above band corresponding to chromosome XI from *S. cerevisiae* has been translocated or deleted in the 10th generation (**Figure 4-2**). As shown in Fig. 2, in generation 1 and 5 of the same colony, this chromosome band is still present, thus the observed chromosomal change has occurred between the 5th to the 10th generation.

Most probably, as one band disappears, a change of size for one of the other chromosomes or an additional band could be expected but this could not be observed for the chromosomes with a smaller size of up to 750 kb. Considering the high intensity of the big chromosomes with size in the range 945 kb – 2200 kb (chromosome XII, IV, XV, VII, XVI, XIII), the "missing" band might have been involved in a recombination event with some of the largest chromosomes. However, in addition to the limitations in the separation of the big size chromosomes, the brightness of the big size chromosome bands in the gel "masks" possible variation in their copy numbers and sizes, and thus further analyses are needed to confirm this.

Both deletion and translocation of chromosomes have earlier been observed for yeast populations (Codon et al. 1998). It has also been demonstrated that chromosome length stability is chromosomal, strain and medium dependent (Sato et al. 2002). Besides the possibility of changes on the individual gene basis, it has also been reported that the length of lager yeast chromosomes can change during successive fermentations (Pedersen 1994). Additionally, the copy number of individual ORFs in lager strains (Bond et al. 2004) revealed that it can vary from one to six. According to the authors, the observed copy number changes occurred at distinct locations on the chromosomes at a strain specific frequency. The authors suggest that their occurrence could be due to intrachromosomal and/or extrachromosomal recombination.

Mainly in the 90s, many reports have focused on the chromosomal changes in both brewing and laboratory yeast strains. Previous study with haploid and diploid populations of *S. cerevisiae* grown on phosphate deficient media, revealed both deletions and increase in chromosomal size involving both putative tandem and non-tandem duplications. For those populations, increase in chromosome size with up to 390 kb has been observed (Adams et al., 1992). Thus, it is reasonable to expect that for allotetraploid strains such as Weihenstephan 34/70, a missing chromosome with a size of 690 kb and 775 kb are either lost or translocated. Most probably the missing chromosome has been translocated to some of the bigger chromosomes regions. Other classes of rearrangements such as inversions might have also occurred, but as they do not necessarily result in size change, they will not always be detected by pulse-field electrophoresis (Adams et al., 1992).

Currently, only three chromosomes, chromosome VII, IX and XV in *S. cerevisiae* remain without reports of chromosomal polymorphism (Boulton and Quain, 2006). Long term culture stability has been previously examined in the work of Casey (1996), producing "chromosomal fingerprint" using Transverse alternating field electrophoresis (TAFE). Periodic chromosomal analysis of Stroh Brewery yeast samples, deposited once a year for a period of twenty seven years, revealed seven different karyotypes. Interestingly, the observed changes were restricted to only four chromosomes, chromosome I, VI, X and XI. The authors conclude that positive selective pressure might be the driving force for these changes and all of these four chromosomes carry genes that are of great importance for the brewer´s yeast fermentation performance such as flocculence (chromosome I-), glycolysis (VI-), maltose utilisation (XI-) and diacetyl production (X-). Coincidentally or not, the observed two chromosomal translocations in the present study have sizes close to chromosomes X and XI from *S. cerevisiae*.

An occurrence of chromosomal rearrangement for 5^{th} or 10^{th} re-inoculation cycles might sound very frequent, however, it should be remembered that within each cycle there are many generations. It should also be noted that the colony generations were cultivated on YPD media, which provides optimum environment for the yeast cells growth. However, during fermentation, the brewer´s yeast is exposed to more stressful environment, in terms of high sugar concentrations, high ethanol concentrations, low temperature and low nitrogen and microelements availability. Thus, it could be expected that in the course of beer fermentation, potential chromosomal rearrangements (especially during high gravity beer fermentations) will commonly occur at higher frequencies.

James et al., 2008 demonstrated that the lager yeast genome shows high plasticity during fermentation. The authors demonstrated that lager brewer´s yeast strain CMBS and its mutants selected at high specific gravity media are undergoing gross chromosomal rearrangements and regional amplification in response to stress. Fermentations of strain CMBS with high gravity wort resulted in greater degree of chromosomal instability and gene amplification, particularly in the telomeric regions, tRNA or ARS elements.

However, it is still not well known whether existence of certain chromosomal translocations, will affect the brewer´s yeast strain phenotypically. In a study of (Sato et al., 2002), assessing the genomic stability of two lager beer yeast strains revealed that genetic alterations were observed for the small chromosomes – chromosome I and VI, after the strains have been propagated for 700 generations. However, the authors proposed that there was no clear relationship between the

brewing yeast fermentation performance and such genetic changes. Microarray based comparative genomic hybridization has been used to study the relative copy number of the individual *S. cerevisiae*-like genes in two lager beer yeast strains in comparison to a haploid laboratory *S. cerevisiae* strain (Bond et al., 2004). It was found that the copy number for large contiguous groups of *S. cerevisiae* genes was similar for both lager yeast strains and the majority of loci where the copy number changes were conserved in both strains. However, the increased copy number of the *S. cerevisiae*-like genes to the right of the *MAT* locus did not result in any significant increase in the gene expression of the studied time-points fermentation. This observation leads to the hypothesis that some form of dosage compensation might take effect in the lager strains.

Our study confirms that brewer´s yeast genome does undergo chromosomal rearrangements in subsequent reinoculation cycles. However, the question whether or not such gross chromosomal rearrangements will influence phenotypically the fermentation performance of brewer´s yeast is still open. Considering the larger genome size of the brewer´s yeast and the hypothesis that some form of dosage compensation effect might take place, it is possible that loss in chromosomal band does not affect phenotypically the brewer´s yeast, its subsequent fermentation performance and the respective quality of the beer product.

References

Adams J., Puskas-Rozsa S., Simlar J, Wilkie C.M. (1992) Adaptation and major chromosomal changes in populations of *Saccharomyces serevisiae*. Curr. Genet. 22:13-19.

Bond U, Neal C, Donnelly D, James T.C. (2004) Aneuploidity and copy number breakpoints in the genome of lager yeasts mapped by microarray hybridization. Curr. Genet. 45:360-370.

Boulton C, Quain D, Brewing Yeast and Fermentation, Blackwell Science Ltd, Oxford, 2006, pp. 29-46.

Casey G (1996) Yeast selection in brewing. In: Yeast strain selection. Chandra J. Panchal (ed.) pp.65-113.

Codon A., Benitez T, Korhola M (1998) Chromosome polymorphism and adaptation to specific industrial environments of *Saccharomyces* strains. Appl Microbiol Biotechnol. 49:154-163.

Dietvorst J, Blieck L, Brandt R, Van Dijck P, Steensma H (2007) Attachment of MAL32- encoded maltase on the outside of yeast cells improved maltotriose utilisation. Yeast 24:27-32.

Goffeau A, Barrel BG, Bussey H et al. (1996) Life with 6000 genes. *Science*. 274:546-567.

Hansen J. and Kielland-Brandt, M. C. in J.H. de Winde, (ed.), *Topics in Current Genetics*, vol. 2, Springer-Verlag, Berlin Heidelberg, 2003, pp.143-164.

James TC, Usher J, Campbell and Bond U. (2008) Lager yeasts possess dynamic genomes that undergo rearrangements and gene amplification in response to stress. Curr. Genet. (2008) 53:139-152.

Kellis M. Patterson N, Endrizzi M (2003) Sequencing and comparison of yeast species to identify genes and regulatory elements. Nature. 423:241-254.

Kodama Y., Kielland-Brandt M. C., Hansen J. in P. Sunnerhagen and J. Piškur (eds.), *Comparative Genomics*, Springer- Verlag, Berlin Heidelberg, 2005, pp. 145-164, DOI 10.1007/b106370.

Meaden P (1990) DNA fingerprinting of Brewer´s yeast: current perspectives. J. Inst. Brew. 96:195-200.

Nakao Y., Kodama Y., Nakamura N., Ito T., Hattori M., Shiba T., Ashikari T., Proc. 29th Congr. Eur. Brew. Conv., Dublin, 2003, pp. 524-530.

Pedersen MB (1994) Molecular analyses of yeast DNA tools for pure yeast maintenance in the brewery. J. Am. Soc. Brew Chem 52:23-27.

Rainieri S, Kodama Y, Kaneko Y, Mikata K, Nakao Y, Ashikari T (2006) Pure and mixed gentic lines of *Saccharomyces bayanus* and *Saccharomyces pastorianus* and their contribution to the lager brewing strain genome. Appl. Environ. Microbiol. 72:3968-3974.

Sato M, Watanari J, Takasio M (2002) Effect of growth media and strains on structural stability in small chromosomes (chromosome I, VI and III) of bottom-fermenting yeast. J Inst Brew 108:283-285.

Sato M., Watanari J., Sahar H., Koshino S. (1994) Instability in electrophoretic karyotype of brewing yeasts. J. Am. Soc. Brew. Chem. 52:148-151.

Smart K.A. (2007) Brewing yeast genomes and proteome profiling during fermentation. Yeast **24**:993-1013.

Tamai Y., Momma T., Yoshimoto H. and Kaneko Y. (1998**)** Co-existence of two types of chromosome in the bottom fermenting yeast, *Saccharomyces pastorianus*. Yeast, Vol. 14, p. 923-993.

Yoshimoto H., Momma T., (1998) Characterisation of the ATF1 and Lg-ATF1 encoding alcohol acetyltransferases in the bottom fermenting yeast *Saccharomyces pastorianus*. J. Ferm. Bioeng. 86:15-20.

5

Transcriptional study of high gravity beer fermentations- the effect of glucose repression and nitrogen limitation

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The content of this chapter forms the basis of manuscript in preparation

Key words: High gravity, brewer´s yeast, transcriptome, glucose repression, nitrogen limitation

Abstract

In the present study, genome wide transcription analyses were used to study the effect of high gravity brewing, achieved with the addition of either glucose or maltose rich sugar syrups on the metabolism of the industrial lager brewer´s strain Weihenstephan 34/70. With increase in the gravity, down regulation of the genes involved in transcription and translation and up-regulation of the genes involved in organelle organization, RNA metabolic process and ribosomal biogenesis were observed. The effect of the growth phase among the studied conditions resulted in higher number of significantly changed genes- 698, compared to the number of significantly changed genes- 19 for the effect of the sugar syrup supplementation. Addition of sugar syrups to increase the gravity clearly shows the effect of nitrogen limitation when comparing the transcriptome profiles of the studied brewer´s yeast from the stationary phase of average (14 °P) and very high gravity (24 °P) fermentations. For the interaction effect of the growth phase and the type of sugar syrup used, higher number of significantly changed genes (both up and down-regulated) for the early exponential and respectively stationary phase of the 14 °P fermentations, compared to the 24 °P fermentations were observed. The significant genes involved in the group “response to stress” for the interaction effect of the growth phase and type of sugar syrup used included genes involved in variety of stresses such as heat shock, DNA repair, oxidative stress, trehalose biosynthesis, osmotic stress, salt stress and autophagy.

Introduction

Today beer is one of the largest beverage products and lager beer production accounts for 90% of the beer being produced. For the last couple of decades, beer has been increasingly produced using innovative technologies. With today's demand to produce quality beers in a short time and in the least expensive way, a majority of the lager beer produced today is a product of high gravity brewing technology. It requires the use of wort with higher sugar concentration and because of the higher ethanol concentrations produced, at the end, the beer is diluted to the desired ethanol content.

During the course of fermentation, brewer´s yeast is exposed to changes in oxygen concentrations, pH, osmotic pressure, ethanol concentrations, nutrient availability and temperature. In high gravity beer fermentations, this stressful environment is further enhanced by the higher glucose concentrations resulting in higher osmotic pressure at the beginning of the fermentation and elevated ethanol concentrations in addition to nutrient starvation toward the end of the fermentation (Piddocke et al., 2009; Blieck, 2007). As a consequence, slower growth rate, reduced viability and vitality, longer lag phase, incomplete (sluggish) fermentation and modified flavor and aroma profile of the final beer can be observed. On industrial scale, brewer´s yeast is reused a number of times in consequent fermentations and the ability of brewer´s yeast to effectively response to these stressful conditions is essential not only for beer production, but also for maintaining the "fitness" of yeast for use in subsequent fermentations (Gibson et al., 2007). A number of studies, involving laboratory or hybrid strains of *S. cerevisiae* on laboratory scale fermentations with minimal or complex media have been used to elucidate the transcriptional response to a number of brewing-relevant parameters as for example- glucose repression, nutrient limitation, variety of stress, anaerobiosis, ageing and ethanol toxicity.

Currently very few studies have utilized lager yeast production strains and used brewing wort as a media to underline the complexity of such system (Gibson et al., 2008). Despite the availability of such transitional data studies, understanding the genome and its expression during the course of fermentation and its contribution to the flavour development remains a key goal in the brewing industry.

However, complication in studying the lager brewer´s yeast transcriptional profile comes from the challenging nature of its genome. Lager brewer´s yeasts belong to the group of *Saccharomyces pastorianus* and it is considered a polyploid species hybrid of *Saccharomyces cerevisiae* and other

closely related *Saccharomyces* species. The average nucleotide percent identity between the two parental species is reported to be 80% in the coding regions and 62% in the non coding regions (Kellis et al., 2003). Due to the complex genome nature of lager brewer´s yeast, associated with its polyploid nature and chromosomal translocations, its sequencing is challenged. The lager strains' specific genes exhibit 85-98% homology to the published sequence of *S. cerevisiae*, while the genes derived from the other parental species have been found to be about 75-85% identical (Kodama et al., 2005). In such cases, for the regions of the non-Sc cerevisiae part and the mosaic part of the genome, DNA from the lager strains do not hybridize to the *S. cerevisiae* DNA arrays and for the probes where hybridization occurs, it has been suggested that the extent of detection will depend on the minor sequence differences. For example, in the study of James et al., 2003, 5180 of the 6300 ORFs available on the gene chips generated readable signals. Another issue that might influence the extent of expression is the ploidity number. However, it has previously been demonstrated that despite the higher copy number of a certain gene, dosage compensation effect might occur in the lager strains, thus the copy number might not influence significantly the strength of expression (Bond et al., 2004).

Despite the challenging nature of the lager brewer´s yeast genome and the complexity of the brewing wort, the use of genome wide transcriptional studies will provide a solid base for the characterisation of the beer fermentation process, for studying the genetics and physiology of the lager brewing yeast strains under controlled environmental conditions, and provide novel opportunities for system wide analyses of the brewer´s yeast complex cellular function. Such knowledge can further be used in brewing fermentation optimisation strategies to characterise the key metabolic pathways and to determine their constraints.

In the current study, genome wide transcription analyses were used to study the effect of high gravity brewing, achieved with the addition of either glucose or maltose rich sugar syrups on the metabolism of the industrial lager brewer´s strain Weihenstephan 34/70. For the fermentations, model fermentation system on laboratory scale was used, designed to simulate as close as possibly the large scale brewing process. Particular focus was given on investigating the brewer´s yeast response in relation to stress and nitrogen metabolism. In addition, the transcriptional profiles from the early exponential and stationary phase of high and low gravity fermentations were compared.

Materials and methods

Strain

The flocculent bottom fermenting industrial lager beer yeast strain Weihenstephan 34/70 (Hefebank Weihenstephan, Freising, Germany) was used in this study. The strain was maintained as a frozen stock culture in 40 % (v/v) glycerol.

Fermentation setup

For the studied fermentations, wort with a gravity corresponding to 14, 21 and 24 °Plato was used. Higher gravity was achieved with either glucose or maltose syrup supplementation to the reference wort of 14 °Plato. The fermentation setup was designed to simulate as closely as possible the larger scale beer fermentations.

Wort

All-malt wort with starting gravity of 14.3 °P and pH=5.2, (Alectia, Denmark), was used. The wort contained 90 % carbohydrates of which the fermentable carbohydrates consisted of 4.4 % fructose, 12.5 % glucose, 66.5 % maltose and 16.7 % maltotriose (w/v). The wort also contained non-fermentable carbon sources such as dextrins and β-glucan. $ZnSO_4.7H_2O$, was added to a concentration of 0.1 ppm Zn. For adjusting the wort to higher gravities- 21° Plato, highly fermentable syrups- Clearsweet® 95% Refined Liquid Dextrose Corn Syrup (95.5 % glucose, 2,5 % maltose, 1 % maltotriose, 1 % higher saccharides, present in % dry basis (w/w) and Satin Sweet® 65 % High Maltose Corn Syrup (70 % maltose, 18 % maltotriose, 9 % higher saccharides, 2 % glucose, present in % dry basis) were used as adjuncts. Both syrups were kindly provided from Cargill Nordic A/S. The resulting sugar composition of the different fermentation media used in this study is summarized in Chapter 3. Prior to inoculation, the wort was oxygenated until it reached 100 % saturation.

Fermentation conditions

For the pre-cultures, the yeast from the stock culture was propagated on YPD plates at 30 °C for four days. A single yeast colony was transferred to 20 ml of 14 °P wort in a sterile 50 ml Falcon tube and incubated at 25 °C in a rotary shaker at 150 rpm. After 48 hours, the preculture was transferred to a 500 ml shake flask with 375 ml of fresh wort and incubated for 72 hours.

All fermentations were performed in 2.2 liter bioreactor (Biostat B5; Braun Biotech International, Melsungen, Germany) with a working volume of 1.5 liter. Dissolved oxygen was monitored with an autoclavable polarographic oxygen electrode. The fermentors were connected with Braun Biotech Multi-Fermenter Control System (MFCS) for data acquisition. Silicone based antifoam agent FD20P at a concentration of 0.1 ml/L (Basildon Chemicals, England) with a food gradient quality was used in the fermentations. The reactors were inoculated with a volume of pre-culture, corresponding to 1×10^7 cells/ml. During the cultivation the temperature was maintained at 14 °C and the stirring was set to 90 rpm. Prior to sampling the stirring was increased to 300 rpm for 2 min. The higher stirring allowed better mixing and homogenization of the media and ensured representative sampling. The pH was recorded on-line, but it was not controlled. After the fermentation was completed, the whole fermentation broth was transferred to a sterile vessel and stored for 14 days at 0 °C, for further maturation. Detailed physiological characterization of cultivations performed at the wort compositions at 14 and 21 °P fermentations has been described previously (Chapter 3).

Transcriptome analysis

Probe preparation and hybridization to arrays

Samples for genome wide expression analysis were taken in duplicates from the stationary phase for each of the fermentations. Samples from the early exponential phase were also collected from fermentations at 14 and 24 °Plato. For each sample, 20 ml of culture were sampled into 50 ml tubes containing 20 ml crushed ice and immediately centrifuged at 4000 rpm for 5 min at 4 °C. The supernatant was discarded and the pellet was frozen instantly in liquid nitrogen and stored at -80 °C. Total RNA was extracted using RNase easy mini kit (Qiagen), according to the protocol for isolation of total RNA from yeast. The quality and the integrity of the extracted total RNA was assessed using the Bioanalyzer 2100 (Agilent Technologies Inc., USA) and RNA 6000 Nano LabChip kit. The cDNA synthesis, cRNA synthesis, labeling and hybridization on the GeneChip® Yeast Genome 2.0 Array (Affymetrix, CA) was performed as described in the Affymetrix GeneChip® expression analysis manual [1]. Washing and staining of the arrays was done using GeneChip® Fluidics Station FS-450 and GeneChip® 3000 7G Scanner was used for scanning.

Data acquisition and gene expression analysis

Affymetrix Microarray Suite v5.0 was used to generate CEL files from the scanned microarrays and the statistical open source language R (Gentleman et al., 2004) was used to perform data analysis. Data preprocessing was carried out using the Robust Multichip Average (RMA) method (Irizarry et al., 2003), available within the R/affy package (Gautier et al., 2004). This package implements RMA by correcting the Perfect Match (PM) probes, performing quantile normalization (Bolstad et al., 2003) and calculating the expression measure by using median polish.

Pair-wise comparisons between conditions were carried out in order to detect differential transcriptional regulation. Moderated t-test implemented in the R/Limma package was used for this purpose (Smyth, 2004). The selected gene lists were used as input for the Reporter Metabolites and Reporter Transcription Factor analysis. Limma package was also used to perform one-way Analysis of Variance (ANOVA) to investigate the effect of the sugar syrup type across 14, 21 and 24 ºP fermentations. *P*-value 0.05 was chosen as cut-off for significance and multiple correction was performed according to Benjamini and Hochberg methodology (Benjamini and Hochberg, 1995).

Two-way ANOVA was used in order to identify significantly changed gene expression levels with respect to the factors characterizing the experimental design: ‘wort type’ and ‘fermentation phase’. The model was fitted to study the effect of these two main factors along with the interaction factor (‘wort type*fermentation phase’). The *p*-values were corrected for multiple testing by applying the False Discovery Rate (FDR) methodology described by Benjamini and Hochberg (Benjamini and Hochberg, 1995) and genes were selected by imposing a cut-off value of 0.05. Furthermore, as regards to the gene lists associated with the two main factors, only genes with $|\log_2(\text{fold change})| > 1.59$ were considered. Genes selected within the interaction term were further investigated in order to select the significant genes unique for each of the possible factor level combinations: ‘14°P wort*early exponential phase’, ‘14°P wort*stationary phase’, ’24°P glucose wort*early exponential phase’, ’24°P glucose wort*stationary phase’, ‘24°P maltose wort*early exponential phase’ and ’24 °P maltose wort*stationary phase’. The identification of these genes was achieved by using the template match method and the R/code described by Pavlidis (Pavlidis, 2003). Moreover, genes selected within the interaction term were clustered (hierarchical cluster with Pearson correlation based distance and average linkage distance measure).

Further on, Gene Ontology (GO) process terms of the selected genes using GO Slim Mapper tools (Saccharomyces Genome Database, SGD) with significance at P <0.01 was investigated.

Reporter metabolites

Using the entire gene expression set, reporter metabolites (algorithm) analysis was used to identify the global metabolic events that significantly responded to the increase in the gravity in the glucose or maltose syrup supplemented fermentations. Reporter metabolites algorithm is based on the reconstructed genome-scale metabolic network of *S. cerevisiae* and it was represented as bipartite indirected graph to link gene expression level to metabolic function and scoring for each metabolite was based on the normalized transcriptional response of its neighbor enzymes (Patil and Nielsen, 2005; Nookaew et al., 2008). The *p* values from the moderate t-test (Limma) performed for each of the pair-wise comparisons of expression data were then used to determine the reporter metabolites.

Clustering analysis

Consensus clustering algorithm with random initialization was used (Grotkjaer et al., 2006) in order to identify profiles of the co-regulated genes. The algorithm was implemented in the MATLAB toolbox ClusterLustre. Pearson correlation was used as a similarity metrics and as a clustering method K-means normalization and accounting for biological replicateswas used. Clustering was repeated 10 times with a new random seed initialization.

Reporter transcription factors identification

Reporter regulators, also named reporter transcription factors or TFs, were determined using the software and regulatory network of Oliveira et al., (2008). The software is based on reconstructed graph covering each known transcription factor or regulatory protein, connected to all genes known to be affected by these proteins from the Yeast Protein Database (YPD). Using the entire gene expression set, reporter regulators analysis was used to identify the transcription factors and their regulatory pathways that were most significantly affected by the increase in the gravity for the glucose or maltose syrup supplemented fermentations.

In brief, for the Reporter TFs algorithm, gene expression data from the significance of change for moderate t-test comparison (Limma) was used as an input together with the topology of the bio-molecular interaction networks (physical or functional interactions) represented as graph, where

each gene of interest is associated with a transcription factor (TFs) (Oliveira et al., 2008). The basic regulatory principle behind is that a perturbation or a response to perturbation might trigger regulatory response beginning from the first neighbors of the affected nodes. The algorithm identifies group of neighbor genes (genes associated with certain feature), that are significantly and collectively co-regulated compared to the background. This concept is extended to any n^{th} degree neighbors. The algorithm does not require accounting for the significant changes at the level of each node (for example, the transcript of a gene). Gene expression, in the form of Z-score is then mapped onto the "gene nodes" of the graph. The score of each feature can be calculated based on the score of its neighbours "gene nodes" and reporter features are then those features with a Z-score above the selected cut-off (Oliveira et al., 2008).

Results

During the course of high gravity beer fermentations, brewer's yeast is exposed to a number of stressful conditions such as high osmotic pressure caused by the high glucose concentrations at the beginning of the fermentations and ethanol stress, imposed by the elevated ethanol concentration levels towards the end of the fermentations. Previously, we have characterized the lager beer yeast strain Weihenstephan 34/70 at average gravity – 14 °P and at high gravity fermentations achieved with either glucose or maltose syrup supplementation at 21 and 24 °P (Chapter 3). When sugar syrups are used as adjuncts to achieve higher gravity, the resulting wort has free amino nitrogen concentrations lower than the minimum required, thus brewer´s yeast is exposed to an additional stress, caused by the induced nitrogen limitation and limitations in other nutrients, resulting in restricted growth (Gibson et al., 2007). The combination of the above mentioned factors lead to a lower specific growth rate, longer lag phase before initiation of ethanol production and incomplete fermentation with higher amount of residual sugars at the end of the fermentations. Additionally, the flavour profile of the final beer was adversely affected by a two to three fold increase in the concentrations of ethyl acetate and isoamyl acetate in the final beer.

The use of maltose syrup instead of glucose syrup to increase the gravity, considering that there was no additional nitrogen supplementation resulted in more balanced fermentation performance in the 21 °P fermentations in terms of higher specific growth rate, respectively higher wort fermentability

and more favored flavour profile of the final beer with lower ethyl acetate, isoamyl acetate and acetaldehyde concentrations.

In order to further investigate the response of the lager brewer´s yeast strain Weihenstephan 34/70 to various stress factors imposed on it during high gravity beer fermentations, we studied the changes in the brewer´s yeast transcriptional response at the early exponential phase and from the stationary phase of the fermentations at average gravity- 14 °P and at very high gravity- 24 °P. Factorial design of 2 x 2 x 2, resulting in 8 different growth conditions was pursued (**Figure 5-1**). The experimental design included gravity, type of sugar syrup used as adjunct to achieve higher gravity and fermentation phase. Each factor included two levels: gravity (14 °P and 24 °P), sugar syrup (glucose and maltose rich syrup) and fermentation phase (early exponential versus stationary phase). Additionally, to investigate the effect of the type of sugar syrups on the brewer´s yeast metabolism, comparative study of the transcriptome samples from the stationary phase of high gravity beer fermentations at 21 °P and 24 °P both with glucose and maltose syrup supplementations were performed. Each of the studied conditions was investigated in duplicates.

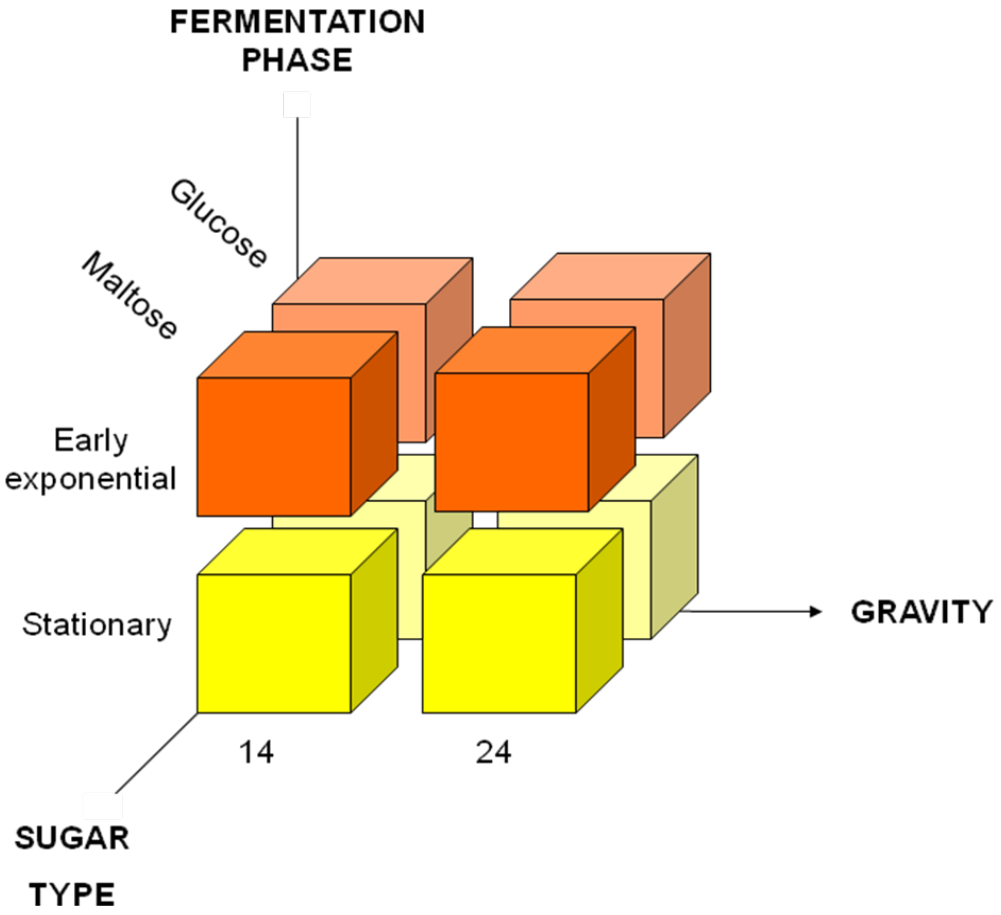

Figure 5-1. **Experimental design**. *Each block represents one of the 8 possible combinations among the three experimental factors (fermentation phase, sugar type and gravity). Each experiment was carried out in duplicate.*

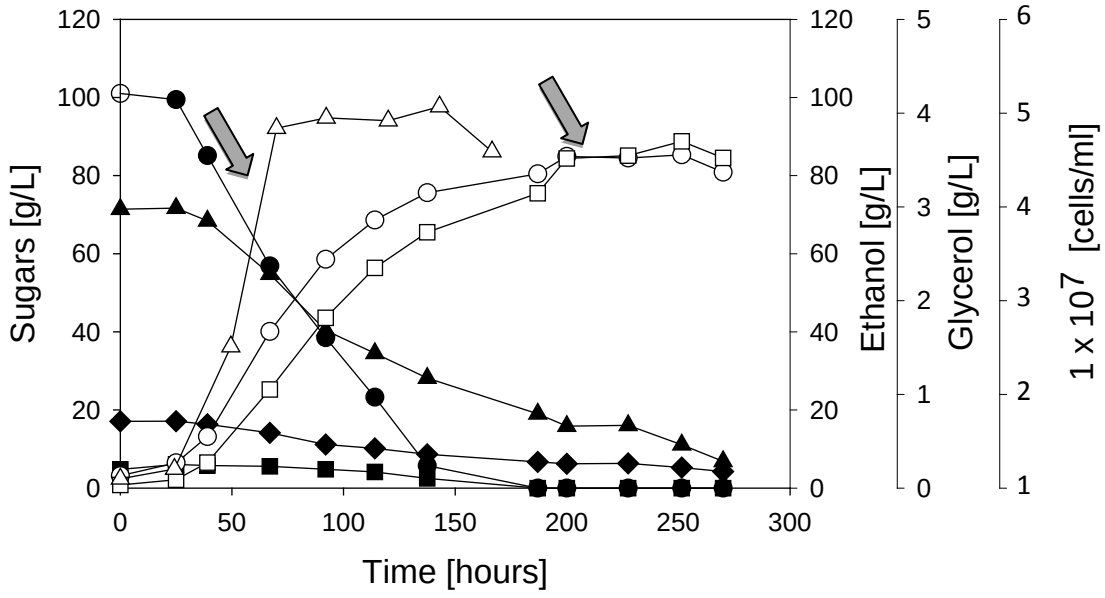

Figure 5-2. Example of the fermentation profile from the studied fermentations. *The arrows in the figure correspond to the sampling points for transcriptome analysis of the studied fermentations. The symbols in the graphs represent the concentrations of: " ● " glucose, " ■ "fructose, " ▲ " maltose, " ◆ " maltotriose, " □ " ethanol," ○ " glycerol and " △ " cells/ml.*

Example of the fermentation profile from the studied fermentations is present in **Figure 5-2** for the 21 °Plato fermentations with glucose syrup supplementation. Detailed physiological characterization of the studied fermentations is present in Chapter 3.

The main products of beer fermentations are ethanol, carbon dioxide, glycerol and yeast biomass. With increase in the gravity, both for the glucose and for the maltose supplemented fermentations, the final cell numbers and the specific growth rate decreased and higher amount of residual sugars remained at the end of the fermentations (Chapter 3). The highest ethanol yield based on consumed sugars was observed for the 21 °P fermentations, followed by the 24 °P fermentations. While for the 21 °P fermentations more complete fermentations in terms of sugar utilization were observed for the maltose supplemented fermentations, for the 24 °P fermentations, both glucose and maltose supplemented fermentations had similar amounts of residual maltose and maltotriose left at the end of the fermentations.

Besides changing the gravity when the sugar syrup content is increased, there is a dilution of free amino nitrogen (FAN) content present in the wort, i.e. the fermentations at 21 and 24 °P had lower initial FAN amounts than the necessary for optimal yeast cell growth and fermentation

performance. However, increasing the gravity by adding maltose syrup as opposed to glucose syrup resulted in more balanced fermentation performance in terms of higher cell numbers, and higher ethanol yield (based on consumed sugars) and a more favorable flavour profile of the final beer in terms of lower concentrations of ethyl acetate and isoamyl acetate compared to the glucose syrup supplemented fermentations.

Effect of the growth phase

Results from the two ways ANOVA revealed 698 significantly changed genes based on the effect of growth phase (early exponential versus stationary) and independently of the other two factors- type of sugar syrups used and gravity of the wort. GO process terms revealed that the main categories to which the significantly changed genes belong and their respective cluster frequencies were: RNA metabolic process (14.3 %), transport (11.2 %), translation (10.2 %), ribosome biogenesis (9.4 %), response to stress and chemical stimulus with respectively (7.7 %) and (7.2%) cluster frequency. List of the GO process term categories to which the significantly changed genes belong with at least 4% of cluster frequency is presented in **Table 5-1**.

The category “response to stress” and examples of the genes involved in them included mainly representatives of the heat shock family (ATG8, HSP26, HSP42, HSP78), DNA repair (APN2, RAD28, REV3), oxidative stress (PRX1, UGA2, ZTA1, GRX1, GRX2, TRX3, TSA2, OXR1, GCY1), trehalose biosynthesis (TPS2), osmotic stress (GRE1), salt stress (HAL1) and autophagy (ATG29).

Table 5-1. GO term for the effect of the growth rate.

GO term (698 genes)	Gene hits	Cluster Frequency
biological process unknown	201	28.80%
RNA metabolic process	100	14.30%
transport	78	11.20%
translation	71	10.20%
ribosome biogenesis	66	9.40%
response to stress	54	7.70%
response to chemical stimulus	50	7.20%
transcription	49	7.00%
carbohydrate metabolic process	47	6.70%
organelle organization	47	6.70%
generation of precursor metabolites and energy	40	5.70%
cell cycle	34	4.90%
cellular amino acid and derivative metabolic process	34	4.90%
protein modification process	33	4.70%
anatomical structure morphogenesis	29	4.10%
lipid metabolic process	26	3.70%
cofactor metabolic process	23	3.30%
cell wall organization	21	3.00%

Clusters represented with at least 3% of cluster frequency are included.

Effect of the type of sugar syrup addition

Considering only the effect of sugar syrup addition used and independently of the other factors, among the 14 and 24 °Plato fermentations, two ways ANOVA analyses revealed 19 significantly changed genes overall at the selected p-value of 0.05 and fold change of 3. Three of them were ORFs with unknown function. GO terms for the main GO process categories for the remaining 16 genes with known function included: transport (36.8%), including BAP2, UGA4, DUR3, HXT1, DAL4, SKS1, OPT2, heterocycle metabolic process (26.3 %), consisting of the group of DAL1-4 genes and DAL 7, DUR1-2 and MTD1 genes, as well as protein modification process (10.5%) cluster frequency. The groups of DAL genes as well as DUR3 genes are involved in nitrogen catabolism and nitrogen catabolite repression (NCR). In particular, the genes DAL 1-4 and DAL7 are involved in the different steps of allantoin degradation and their expression is sensitive to nitrogen catabolite repression.

In addition, to investigate independently the effect of either glucose or maltose syrup supplementation at the stationary phase of the studied fermentations, one way ANOVA analysis, including also samples from the fermentations at 21 °P where performed. Based only on the effect of the type of sugar syrups used across the 14, 21 and 24 °P fermentations and independently of the gravity, Limma analyses revealed only 20 significantly changed genes for the glucose supplemented fermentations with a cut off p-value of 0.05. Gene ontology terms revealed that the main categories to which they belong and their represented cluster frequency were: unknown biological process (40%), transport (25%), as well as organelle organization, RNA metabolic process, transcription, vitamin and cofactor metabolic process, each involving 2 genes (10% of cluster frequency) (**Table 5-2**). However, for the maltose syrup supplemented fermentations, at the selected cut off p-value of 0.05, Limma analyses showed 1530 genes significantly affected by the addition of maltose syrups. GO terms for the main biological process categories involved in this group revealed that 30% of them are involved in organelle organization, 19.8% were involved in unknown biological process, 16.7% in RNA metabolic process, 14.4% in transport, 12.9% in translation and 9.2% in ribosome biogenesis (**Table 5-2**). In total, 124 genes, representing 8.1% of cluster frequency were involved in the category "response to stress". The main groups of stress for the significantly changed genes in this category where: DNA repair (HTA2, RAD57, RAD28, RAD34, OGG1, MSH2, RAD50, POL2, MSH2), cellular stress response to DNA damage- TOS4, biogenesis and maintenance of mitochondria (PIM1), oxidative stress (UGA2, SCO1, GRX4, TSA2), heat shock proteins (HSP42), response to hyperosmotic stress (HOG1, GRE3, DDR48), cold shock and anaerobiosis (TIR2), autophagasome formation *(ATG1,* ATG7), glucogen synthase (YGK3).

Table 5-2. GO terms based on biological processes ontology for the individual effect of the glucose rich or maltose rich syrups used.

Glucose syrup supplementation			Maltose syrup supplementation		
GO terms (20 genes)	Number of genes in the cluster	Cluster Frequency (%)	GO term (1530 genes)	Number of genes the cluster	Cluster Frequency (%)
biological process unknown	8	40%	organelle organization	459	30%
transport	5	25%	biological process unknown	303	19.8%
organelle organization	2	10%	RNA metabolic process	256	16.7%
RNA metabolic process	2	10%	transport	221	14.4%
transcription	2	10%	translation	198	12.9%
vitamin metabolic process	2	10%	ribosome biogenesis	140	9.2%
cofactor metabolic process	2	10%	protein modification process	131	8.6%
protein modification process	1	5%	cell cycle	127	8.3%
carbohydrate metabolic process	1	5%	response to stress	124	8.1%
sporulation resulting in formation of a cellular spore	1	5%	transcription	122	8%
DNA metabolic process	1	5%	DNA metabolic process	106	6.9%
generation of precursor metabolites and energy	1	5%	response to chemical stimulus	86	5.6%
translation	1	5%			

Clusters represented with at least 5% of cluster frequency are included.

Interaction effect of both terms

Considering the interaction effect of both the fermentation phase and the type of sugar syrups used among the 14 and 24 °P fermentations (**Figure 5-3**), 129 genes were significantly changed (**Table 5-1S**, supplementary information). GO terms revealed that the main categories in which the significantly changed genes were involved are: transport (21.7%), organelle organization (20.2%), protein modification process (11.6%), RNA metabolic process (10.9%), transcription and translation, each with 7.8% of cluster frequency. Other groups of interest were carbohydrate metabolic process (6.2%), response to stress (5.4%) and cellular amino acids and derivative metabolic process (4.7%). The genes included in the response to stress group were SCO1 and GPX2- involved in oxidative stress, SHU2 and EAF6- involved in homologous recombination repair, STM1 involved in TOR signaling pathway and respectively nutrient stress and ISC1 and YDC1, involved in sphingolipid metabolism and contributing to variety of stresses.

The significant genes involved in the amino acid metabolism included: MET1, MET6 and ADI1, involved in the methionine metabolism, MSE1 – mitochondrial glutamyl-tRNA synthetase, PUT4- proline oxidase and CAR1- arginase, responsible for arginine degradation, which expression responds to both induction by arginine and nitrogen catabolite repression and its disruption enhances freeze tolerance.

To determine significantly enriched Gene Ontology (GO) process terms within the up-regulated and down-regulated gene cluster of the studied conditions, Saccharomyces Genome Database (SGD)-GO tools (significance at $P < 0.01$) was used. The main GO process term categories for each of the corresponding groups from the templetate match analysis are present in **Table 5-2S** (supplementary information).

The 14 °P fermentations resulted in higher number of significantly changed genes compared to the higher gravity fermentations. Results from the template match analysis showed 26 genes significantly expressed for the exponential growth phase of the 14 °P fermentations. Among those, 13 were up-regulated and 13 were down-regulated. The most overrepresented GO term categories among the 14 °P down-regulated genes were protein modification (46.2 %), carbohydrate (38.5 %) and lipid (15.4 %) metabolic processes. Among the 14°P up-regulated genes, the most overrepresented GO terms were RNA metabolic process (23.1 %), as well as again lipid metabolism (15.4 %) and protein modification process (15.4 %). Four genes from this group also belonged to

unknown biological process categories. For the early exponential phase of the 24°P Gl fermentations, among the 12 significantly changed genes, 9 genes were down-regulated and 3 genes were up-regulated. The 9 down-regulated genes were involved in transport (ADP1, PIC2, DNF3), unknown biological process (CPR4, COS4, AIM17), response to oxidative stress and lipid metabolic process (YDC1) as well as membrane organization (DNF3). The up-regulated genes were involved in ribosome biogenesis (NOP14), response to oxidative stress and chemical stimulus (GPX2), RNA metabolic process (NOP14) and unknown biological process (BSC1). There were no significantly changed genes specific for the early exponential phase of the 24°P M fermentations. For the stationary phase of the studied 14 ^{o}P fermentations, 41 genes were significantly changed. Among those, 21 genes were down-regulated and 19 genes were up-regulated. The main GO process categories for the down-regulated genes included unknown biological process (33 %), cellular amino acids and derivatives (14 %), RNA and DNA metabolic processes, transcription, vitamin metabolic process, response to stress and cofactor metabolic process, each represented with (10 %) of cluster frequency. The genes involved in amino acid metabolic processes were ADI1, PUT4 and CAR1. Gene ADI1 encode acireductone dioxygenease, it is involved in the methionine salvage pathway and its mRNA is induced in heat shock conditions. PUT4 is proline permease and it is involved in proline catabolism and neutral amino acids transport. CAR1 is arginase, responsible for arginine degradation and its expression responds to both induction by arginine and nitrogen catabolite repression. The genes included in the stress response group were SHU2, involved in homologous recombination repair and hyperosmotic stress resistance and EAF6, involved in histone acetylation, respectively. For the stationary phase of the 24 °P M fermentations, there were only 2 significantly changed genes, unique for the maltose syrup supplemented fermentations and none for the glucose syrup supplemented fermentations. The significantly changed down-regulated genes for the 24 °P maltose supplemented fermentations, were respectively PRE5 with endopeptidase activity and RIT1 with transferase activity which modifies the initiator methionine tRNA.

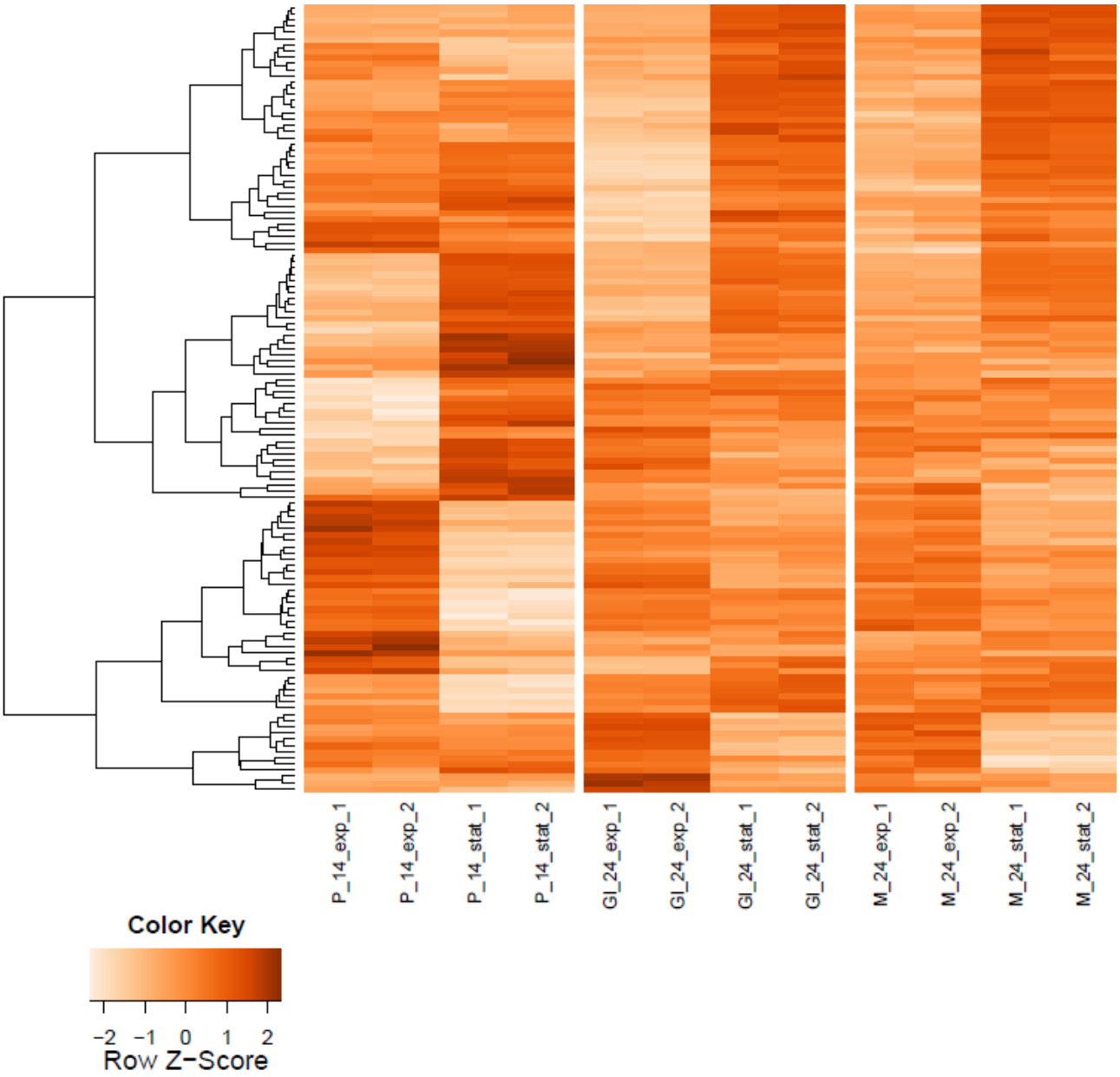

Figure 5-3. Hierarchical clustering of the genes selected within the interaction term. *The number 14 and 24 correspond to the gravity of the wort; Numbers 1 and 2 represent replicate experiments from the same conditions. Gl and M represents glucose and maltose syrup supplementation, respectively. The abbreviations "exp" states for the early exponential phase and "st" for the stationary phase of the samples.*

To determine significantly enriched Gene Ontology (GO) process terms within the up-regulated and down-regulated gene cluster of the studied conditions, Saccharomyces Genome Database (SGD)-GO tools (significance at $P < 0.01$) was used. The main GO process term categories for each of the corresponding groups from the templetate match analysis are present in Table 5-2S (supplementary information).

Cluster luster

Consensus clustering algorithm (Grotkjaer et al., 2006) was used to cluster genes that were co-regulated among the studied conditions. While clusters 3, 6, 7, 10, 11 and 16 had a unique profiles, clusters 1-2, 4-5, 8-9 and 12-15 had similar patterns.

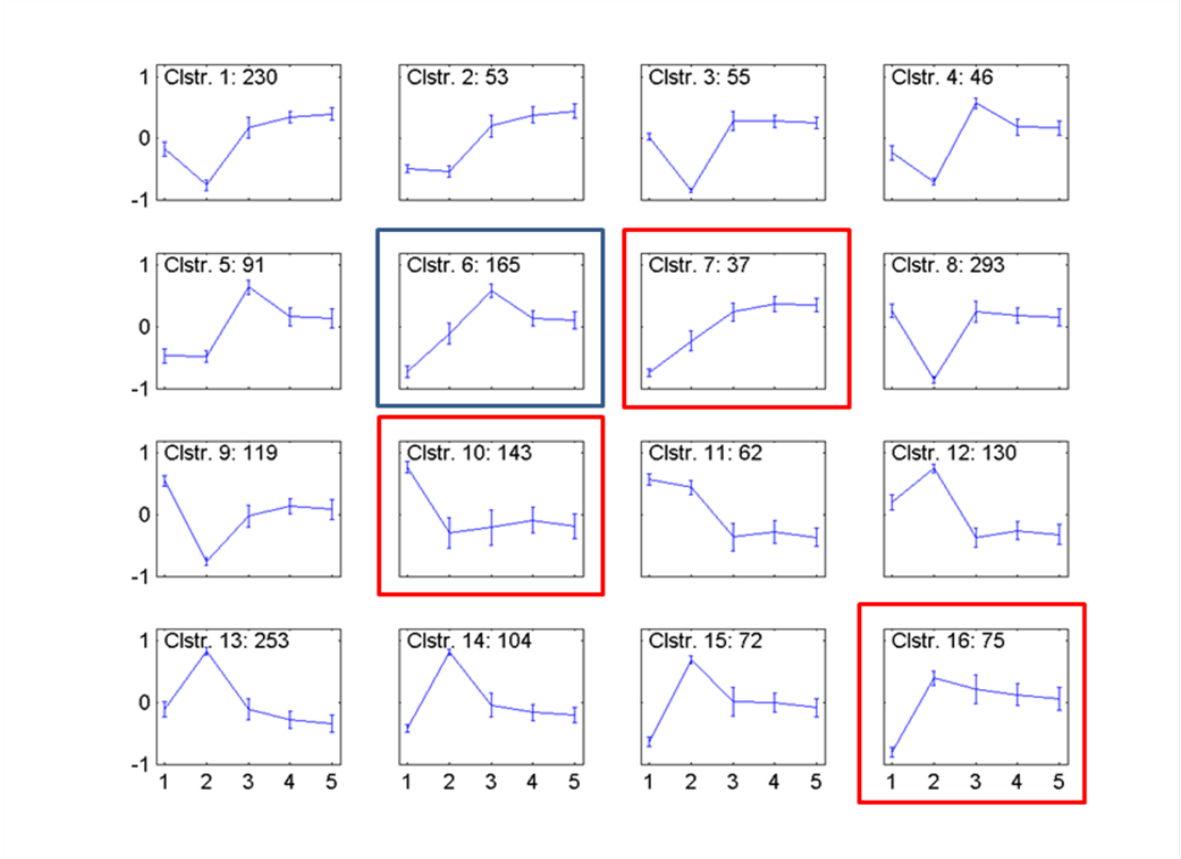

Figure 5-4. Clustering of the significantly changed genes based on the two ways ANOVA of the effect of sugar syrups used and gravity strength. *In total, 16 clusters of genes were identified: The x axis represents the samples at the different gravities from the glucose and maltose syrups supplemented fermentations from the stationary phase: 1 – 14 °P; 2 – 21 °P glucose; 3 –21 °P maltose; 4- 24 °P glucose ; 5 –24 °P maltose, the y axis represents normalized gene expression intensities in the range from -1 to 1.*

Of particular interest in the consensus cluster analysis were the clusters associated with genes up- or down-regulated with increase in the gravity. In the present study, Cluster 10, included 143 genes, down-regulated with increase in the gravity (**Table 5-3S**, supplementary information). The main GO process terms for the genes included in cluster 10 were growth related processes such as transport (14.75 %), transcription (11 %), RNA metabolic process (10 %) and organelle organization (9 %). In addition, the GO terms for the significantly changed genes in cluster 10

included response to stress and amino acids derived metabolism. The genes included in the group response to stress included genes involved in response to oxidative stress (for example- YBL055C, TSA2,ASK10), SBP1- negative regulator of translation in response to stress, ATG7- involved in autophagy, RSC9- which is component of the RSC chromatin remodeling complex and DNA-binding protein involved in the synthesis of rRNA and in transcriptional repression and activation of genes regulated by the Target of Rapamycin (TOR) pathway as well as RIM11- protein kinase required for signal transduction during entry into meiosis. The genes involved in the amino acid metabolism were general amino acid permease (GAP1), genes involved in histidine biosynthesis (HIS7), methionine synthase (MES1, MET6), GABA catabolic process (UGA3), proline catabolism (PUT2) and asparagine synthetase (ASN1). Full list of the GO terms for the significantly changed genes from cluster 10 and their corresponding cluster frequency are present in **Table 5-3S**, supplementary information.

Cluster 7, consisting of 37 genes and cluster 16 consisting of 75 genes, included the genes up-regulated with increase in the gravity from 14 °P through 21 °P to 24 °P. The main GO process terms for the significantly changed genes from cluster 16 besides organelle organization (49.3%), RNA metabolic process (49.3%), ribosome biogenesis (26.7%), as well as transport (16%) and translation (10.7%) were also response to stress (8%), membrane organization and vesicle mediated transport, both with 4.3% cluster frequency (**Table 5-4S**, supplementary information). The genes in group response to stress included: PIM1, involved in proteolysis of mitochondria; genes EAF5, MMS21, SMC5, involved in DNA repair; gene SGD1, encoding essential nuclear protein with a possible role in the osmoregulatory glycerol response and gene ATG17 involved in peroxisome degradation and autophagy.

Full list of the GO terms for the significantly changed genes from cluster 16 and their corresponding cluster frequency are present in **Table 5-5S**, supplementary information.

As expected by the similar normalized gene expression profile across the different gravities (**Table 5-4S**), the main GO process term categories for cluster 7 where very similar to the GO process term categories of cluster 16 (**Table 5-5S**, supplementary information). Most of the genes included in the group response to stress for cluster 7 where also similar to those included in the response to stress group of cluster 16. Unique for the response to stress group on cluster 7 included HTA2- core histone protein required for chromatin assembly and chromosome function, SHO1- transmembrane osmosensor which participates in activation of both the Cdc42p- and MAP kinase-dependent

growth and in the high-osmolarity glycerol response pathway, ELM1- serine/threonine protein kinase that regulates cellular morphogenesis, SMC6- protein involved in structural maintenance of chromosomes and IQG1, essential protein required for determination of budding pattern and involved in osmotic stress. Full list of the GO terms for the significantly changed genes from cluster 7 and their corresponding cluster frequency are present in Table 5, supplementary information.

Of special interest is also cluster 6. Its normalized expression profile showed increase in the expression from 14 towards 21 °P fermentations both with glucose and maltose syrup supplementation, followed by a slight decrease in the 24 °P fermentations. More then half of the genes in this cluster where involved in organelle organization (58.2 %), followed by translation (35.2%), transport (15.8%), unknown biological process (13.9%) and RNA metabolic process (11.5%) (**Table 5-6S**, supplementary information)

For this cluster, the group response to stress included: SCO1- Copper-binding protein of the mitochondrial inner membrane, required for cytochrome C oxidase activity and respiration, AFG1 involved in oxidative stress and YKL069W- Methionine-R-sulfoxide reductase. The genes involved in the amino acids metabolism where: MSW1- Mitochondrial tryptophanyl-tRNA synthetase, NAM2- Mitochondrial leucyl-tRNA synthetase and MSD1- Mitochondrial aspartyl-tRNA synthetase.

Transcription factors and reporter metabolites

To identify and score TFs, that might regulate the most significant changes between the early exponential and stationary phase for the different gravities and between the average (14 °P) and high gravity (24 °P), respectively at their early exponential and stationary phases, we scored the overlap between significant genes from each of the t-test comparisons (**Table 5-3A, Table 5-3B** and **Table 5-4**) and known TF target sets, using hypogeometric test at $P<0.01$ (Oliveira et al., 2008).

Among the significant TF´s for the genes from the t-test comparison of the early exponential and stationary phase for 14, 24Gl and 24M fermentations, the TFs- MSN2, MSN4, OAF1, and PIP2 appeared to be common in all three cases. The TFs YAP1 and SLY1 were common for the fermentations at 14 °P and 24 °P Gl.

MSN2 and MSN4 are homologous and belong to the family of the C_2 H_2 zinc finger proteins (Gancedo et al., 1998). They act as activators of the transcription for genes involved in the response to different types of stress by binding DNA at stress response elements of responsive genes and thus inducing their gene expression. They have also proven role in increased resistance to stress (Martínez-Pastor et al., 1996). The transcription factor OAF1 activates genes involved in beta-oxidation and YAP1 is ferredoxin of the mitochondrial matrix required for formation of cellular iron-sulfur proteins. The TFs- TUP1 and RGT2 where common for the t-test comparison of 14 °P and 24 °P Gl or respectively of 14 °P and 24 °P M at the early exponential phase of the fermentations. TUP1 is a general repressor of transcription, forms complex with Cyc8p, it is involved in the establishment of repressive chromatin structure through interactions with histones H3 and H4, and enhances expression of some genes, predicted to be involved in a stress response. RGT2 is a plasma membrane glucose receptor, highly similar to Snf3p; both Rgt2p and Snf3p serve as transmembrane glucose sensors generating an intracellular signal that induces expression of glucose transporter (HXT) genes. Additionally, the TFs - DAL80, DAL81, DAL82, GAT1, GLN3, SSY1, SWI6, URE2 and VID30, involved in the nitrogen metabolism and nitrogen catabolite repression were common for the t-test comparison between the 14 °P and respectively 24 °P Gl and 24 °P M fermentations at their stationary phase. While DAL80 is a negative regulator, DAL81 and DAL82 are positive regulators of genes in multiple nitrogen degradation pathways and allophanate inducible genes, respectively. GAT1 and GLN3 are transcriptional activators of the genes involved in nitrogen catabolite repression and their activity and localization are regulated by the nitrogen catabolite repression (NCR) and by the quality of the nitrogen source. The transcriptional regulator URE2 is also involved in nitrogen catabolite repression that acts by inhibition of GLN3 transcription. It is also involved in oxidative stress. The TFs SSY1 is component of the SPS plasma membrane amino acid sensor system, which senses external amino acid concentration and transmits intracellular signals that result in regulation of expression of amino acid permease genes. The transcription cofactor SWI6 is involved in meiotic gene expression, VID30 is a protein involved in proteasome-dependent catabolite degradation of fructose-1,6-bisphosphatase (FBPase), it binds FBPase and shifts the balance of nitrogen metabolism toward glutamate production.

The TF´s unique for the glucose supplemented fermentations (t-test comparison between the early exponential and stationary phase of the 24 °P Gl fermentations), were MBP1, RIM 101, SIR3, SAS2 and SLY 1. All of those TFs are involved in nitrogen metabolism- mainly nitrogen catabolite repression, as well as amino acid sensing and oxidative stress. Transcription factor MBP1 is

involved in regulation of cell cycle progression from G1 to S phase, RIM101 is transcriptional repressor involved in response to pH and in cell wall construction as well as negative regulation of transcription from RNA II polymerase promoter, SIR3 is involved in chromatin silencing and SLY1 is a hydrophilic protein involved in vesicle trafficking between the ER and Golgi.

The TFs unique for the maltose supplemented fermentations (t-test comparison between the early exponential and stationary phase of the 24 °P M fermentations) were GZF3, PTR3, RAS2, RPM2 and TOR1. Their main functions are involvement in the control and regulation of nitrogen metabolism- GZF3, PTR3, RAS2, or involvement in transcription and cell cycle processes- RPM2 and CLN3. Other important TF was TOR1. It is the well known PIK-related protein kinase and rapamycin target, it is subunit of the TORC1 complex that controls growth in response to nutrients by regulating translation, transcription, ribosome biogenesis, nutrient transport and autophagy.

RAS2 is a GTP-binding protein that regulates the nitrogen starvation response, sporulation, and filamentous growth, CLN3 is a G1 cyclin involved in cell cycle progression, PTR3 is involved in response to amino acid stimulus and RPM2 is a protein subunit of mitochondrial RNase P, with role in nuclear transcription, cytoplasmic and mitochondrial RNA processing, and mitochondrial translation.

Table 5-3A. Reporter transcription factors from the pair-wise comparisons between the average and high gravity fermentations at their early exponential phase.

	Average vs High gravity					
	14 exp vs 24 GL exp			14exp vs 24M exp		
	Transcription factors	Number of neighbors	P-value	Transcription factors	Number of neighbors	P-value
Early exponential phase	MSN2	61	0.000471	BAS1	16	0.000191
	MSN4	55	0.001648	***RGT2***	6	0.024684
	TUP1	46	0.003804	PHO2	24	0.02841
	SIN4	26	0.017322	***TUP1***	46	0.038699
	ROX1	25	0.003498	RGT1	8	0.042735
	HAP1	24	0.023605			
	MIG1	23	0.011685			
	MOT3	19	0.027335			
	UPC2	17	0.009085			
	SSY1	17	0.001922			
	MET4	13	0.004488			
	SLY1	12	0.045928			
	CBF1	12	0.03071			
	PGD1	11	0.029579			
	ARG82	8	0.04368			
	STD1	6	0.046483			
	SPT7	6	0.039905			
	RGT2	6	0.01389			
	MED6	5	0.04181			
	RGR1	5	0.04181			
	PKC1	5	0.00661			

TFs present in bold and underlines are present in both studied conditions for the early exponential phase;

Table 5-3B. Reporter transcription factors from the pair-wise comparisons between the average and high gravity fermentations at their early exponential phase.

	Average vs High gravity					
	14 st vs 24 Gl st			14 st vs 24M st		
	Transcription factors	Number of neighbors	P-value	Transcription factors	Number of neighbors	P-value
Stationary phase	**SWI6**	32	0.002939	CLN3	6	0.019497
	GLN3	26	8.15E-07	**DAL80**	21	3.77E-06
	MBP1	23	0.013418	**DAL81**	12	3.86E-05
	DAL80	21	5.01E-07	**DAL82**	8	0.000879
	SSY1	17	0.000146	**GAT1**	9	0.018884
	RIM101	13	0.014784	**GLN3**	26	7.02E-06
	DAL81	12	7.52E-06	GZF3	4	0.01775
	SIR3	12	0.007683	PTR3	5	0.006869
	SLY1	12	0.026231	RAS2	20	0.031988
	SAS2	10	0.002502	RPM2	6	0.001921
	VID30	10	0.007619	**SSY1**	17	0.00082
	URE2	9	0.004075	**SWI6**	32	0.009233
	GAT1	9	0.008964	TOR1	4	0.044752
	DAL82	8	4.53E-05	**URE2**	9	0.007823
	RPM2	6	0.000101	**VID30**	10	0.016896
	RFA1	6	0.02185			
	RFA3	6	0.02185			
	PGS1	6	0.026577			
	PTR3	5	0.002155			

TFs present in bold are present in both of the studied conditions at the stationary phase.

Table 5-4. Reporter transcription factors from the pair-wise comparisons between the average and high gravity fermentations at their stationary phase.

Early exponential vs stationary phase								
14 exp vs 14 st			24Gl exp vs 24Gl st			24M exp vs 24M st		
Transcription factors	Number of neighbors	P-value	Transcription factors	Number of neighbors	P-value	Transcription factors	Number of neighbors	P-value
MSN2	61	2.55E-05	***MSN2***	61	0.000142	***OAF1***	13	0.002331
MSN4	55	2.83E-05	***OAF1***	24	0.000236	***PIP2***	13	0.002331
SLY1	24	0.002522	***PIP2***	24	0.000328	***MSN2***	23	0.002394
IFH1	12	0.002553	***MSN4***	55	0.000386	STD1	5	0.003065
PIP2	17	0.00731	RAS2	20	0.005439	***MSN4***	21	0.003811
OAF1	51	0.007939	SLY1	12	0.010501	PKC1	4	0.00454
SNF7	24	0.00794	HSF1	37	0.013575	GRR1	5	0.007133
RFA1	11	0.010912	HAP2	28	0.014186	ISW2	3	0.007345
CAT8	20	0.020796	THI3	9	0.019084	ITC1	3	0.007345
SSY1	24	0.031818	SKN7	24	0.020225	RGT2	3	0.009514
YAP1	20	0.036937	HAP3	25	0.020846	AMA1	3	0.009663
			YAP1	51	0.023675	UME6	10	0.016648
			THI2	8	0.033596	SSN6	6	0.020847
			DAL81	12	0.038019	MIG1	8	0.027586
			HAP5	22	0.04012	SNF4	4	0.041607
			HAP4	26	0.046719			

TFs present in bold are present in all three of the studied conditions, TFs which are underlines are present in two of the studied conditions.

To identify the metabolites in the studied brewer´s yeast around which the expression changes in mRNA were the most significantly associated, we applied the Reporter Metabolite Algorithm (Patil and Nielsen, 2005). The most significant Reporter metabolites based on a t-test comparison, with Z score > 1.8 and number of neighbors >3 are listed in **Table 5-7S** and **Table 5-8S** (supplementary information).

Comparison of the reporter metabolites from the early exponential and stationary phase at 14 °P, 24 °P Gl and 24 °P M fermentations as well as across the average – 14 °P and high gravities- 24 °P Gl and 24 °P M at their corresponding growth phase (early exponential or stationary) was done. Similarly to the TFs reporter features, the reporter metabolites algorithm also confirmed that the significant reporter metabolites when comparing samples from the early exponential and stationary phase for 14 °P and 24 °P fermentations with glucose and maltose syrup supplementation, as well as for the early exponential phase and respectively stationary phase when comparing 14 °P and the 24 °P fermentations were involved in carbohydrate metabolism (glucose, maltose, fructose), response

to stress (oxidative stress- H_2O_2, ergosterol and accumulation of trehalose and glycogen and response to variety of stresses) as well as nitrogen metabolism (ammonia, most of the amino acids, as for example methionine, leucine, cysteine, tyrosine and tryptophan) (**Table 5-7S**, **Table 5-8S**, supplementary information).

Discussion

During high gravity brewing the yeast encounters multiple stress conditions simultaneously and responds to it by adjusting its global transcript gene expression profile (Olesen et al., 2002; James et al., 2003). Yeast cells can rapidly adapt their physiology to prevailing surrounding by reorganizing their genomic expression and so changing the patterns of cellular proteins and metabolites (Gasch and Werner-Washburne, 2002). In the present study, with increase in the gravity, consensus clustering revealed down regulation of the genes involved in transcription, translation and up-regulation of the genes involved in organelle organization, RNA metabolic process and ribosomal biogenesis.

The effect of the growth phase resulted in higher number of significantly changed genes- 698, compared to the number of significantly changed genes- 19 for the effect of the sugar syrup supplementation. The group of the significant genes involved in the GO category “response to stress” for the effect of the growth phase included genes involved in variety of stresses such as heat shock, DNA repair, oxidative stress, trehalose biosynthesis, osmotic stress, salt stress and autophagy. Of those, the largest group of genes was involved in oxidative stress.

In *S. cerevisiae*, two major stress response pathways exist. One of them is the heat shock response (HSR), which is activated by sublethal heat stress and mediated by the heat shock transcription factor (HSF) (Gibson et al., 2007). It has previously been shown that at the late stages of beer fermentation, the expression of the major stress response gene Hsp104 and other heat shock responsive genes are repressed at the end of the fermentation (James et al., 2003). Such results suggest that conditions experienced in industrial brewing prevent the activation of the HSR stress response (Brosnan et al., 2000).

Another stress response pathway is the general (global) stress response (GSR), which is activated by a number of environmental stresses including oxidative, pH, heat and osmotic stresses as well as

nitrogen starvation. It is believed that GSR is an evolutionary adaptation that allows yeast to respond to adverse environmental conditions in a nonspecific manner, in order to maintain the cellular integrity and function whilst specific responses are activated (Martinez-Pastor et al., 1996). It involves the up-regulation of approx. 200 genes and their corresponding proteins, which are involved in wide range of cellular functions (Causton et al., 2001). The expression of these genes is controlled by their stress response elements (STRE). The activation of the STRE element of inducible genes is dependent upon two zinc finger transcriptional activators- Msn2p and Msn4p (Treger et al., 1998) and they are active under wide variety of stresses (Hofmann, 2002) including those prevailing under brewing fermentation conditions and diauxic shift (Boy-Marcotte et al., 1998). Following the stress response, the Msn2p is rapidly degraded (Bose et al., 2005). In the present study, MSN2 and MSN4 where present as a significant transcription factors in the t-test comparisons of the early exponential and stationary phase in all three cases of 14, 24Gl and 24M fermentations. The majority of genes included in the group response to stress for these groups comparisons where also involved in oxidative stress response and reporter metabolites from those studied conditions included ergosterol.

Previous transcriptome studies in high gravity beer fermentations also revealed strong expression of genes involved in the biosynthesis of ergosterol and oxidative stress protection during the early stages of industrial lager beer fermentation (Higgins et al., 2003; James et al., 2003; Olesen et al., 2002).

In addition, for the interaction effect of the growth phase and the type of sugar syrup used, the template match analysis revealed higher number of significantly changed genes (both up and down-regulated) for the early exponential and respectively stationary phase of the 14 °P fermentations, compared to the 24 °P fermentations. Possible reason for such effect is that on one side there is large similarity in the transcriptional profile for the 24 glucose and maltose syrup supplemented fermentations and on the other side that many of the metabolic processes in brewer´s yeast are slowed down, or completely inactive in the conditions of high gravity brewing.

Additionally, consensus clustering analysis also revealed up-regulation of genes involved in the high osmolarity glycerol (HOG) pathway. The HOG pathway is also required for the activation of the Msn2p and Msn4p (Hofmann, 2002), thus suggesting that Hog1p may have a role in regulating the general stress response via the STREs (Schüller et al., 1994).

For the effect of sugar syrup addition, the addition of maltose syrup as adjunct resulted in significantly higher number of significantly changed genes- 1530 compared to the glucose syrup supplemented fermentations. Presence of high concentrations of glucose in the growth medium represses the transcription of multiple genes involved in the alternative carbohydrate and mitochondrial metabolism. This phenomenon is known as carbon catabolite repression (CCR). CCR encounter coordinated downregulation of the transcription of large group of genes involved in metabolism of non-glucose carbon sources, a number of hexose transporters and respiration (Gancedo, 1998). Major role in the global regulation of CCR is played by the two nutrient signaling transducers- SNF1 and GCN2. TOR1 is another important nutritional transducer which has been implicated in the up-regulation of the general amino acid permease with broad specificity- GAP1, in the down regulation of the tryptophan and tyrosine permease- TAT2 and the high affinity histidine permease- HIP1. Despite the large difference in the number of the significantly changed genes in the glucose versus maltose supplemented fermentations, the main GO process term categories where the same- organelle organization, RNA metabolic processes, transport, unknown biological process.

Moreover, the largest group of genes for the interaction effect included in significantly enriched GO processes has no known function. As it has been suggested in a previous study with wine strain of *S.bayanus* at 15 °C fermentations (Pizarro et al. 2008), GO terms unknown processes includes genes possibly involved in adaptation for growth in suboptimal temperatures. Another interesting observation for the genes up-regulated with increase in the gravity (cluster 6) was the observed signifuicantly enriched GO term category cellular respiration, covering nearly 8% of cluster frequency. It has been suggested that the brewing yeast attempt to alter its metabolism to adapt to respiratory growth in response to depletion of glucose and fructose, and possibly, increased ethanol concentrations prior to the loss of fermentable carbohydrates from the system (Gibson et al., 2008).

In *Saccharomyces cerevisiae*, the expression of all known nitrogen catabolite pathways are regulated by four regulators – Gln3, Gat1, Dal80 and Deh1 and their regulation is known as nitrogen catabolite repression (NCR). While Gln3 and Gat1 are positive regulators of the nitrogen catabolite gene expression, Dal80 and Deh1 are negative regulators, down regulating the Gln3 and Gat1 dependent transcription (Hofman-Bang, 1999). In the present study, addition of sugar syrups to increase the gravity clearly shows the effect of nitrogen limitation when comparing the

transcriptome profile of the studied brewer´s yeast from the stationary phase of 14 °P and 24 °P fermentations.

Trehalose is an important stress protectant ensuring proper folding (Singer and Lindquist, 1998) and repair of proteins (Simola et al., 2000). Reporter Metabolites analysis included trehalose as one of the significantly changed metabolites from the t-test comparison of the early exponential and stationary phase of the high gravity fermentations- 24Gl and 24M fermentations. The levels of trehalose are related to stress response and adaptation (Conlin and Nelson, 2007) and trehalose has been reported to be accumulated in response to heat shock, exposure to toxic chemicals, ethanol stress osmotic stress and oxidative stress (Gibson et al., 2007; Pedreño et al., 2002). The genes involved in trehalose synthesis and degradation are also regulated by the STRE elements (Winderickx et al., 1996). Intracellular trehalose has also been shown to be accumulated in response to nutrient depletion (Gibson et al., 2007). Nitrogen limitation induces GSR in yeast (Marchler et al., 1993) and it is possible that accumulation and degradation of trehalose is an STRE-mediated response to nitrogen limitation.

The TF factors from the t-test comparison of the early exponential and stationary phase of the 24Gl fermentations also included the TFs regulating the HAP complex (**Table 5-7**S, supplementary information). The HAP 2/3/4/5 complex regulates a large number of genes. It activates transcription when yeast grows on non-fermentable carbon sources, and presence of glucose or other repressing sugars interfere with its activation (Gancedo et al., 1998). It has been suggested that HAP 2/3/4/5 complex plays a role in remodeling of the chromatin structure, which does not require Hap4 and direct activation of the RNA polymerase, in which Hap4 is involved (Shaw et al., 1996).

Despite the large similarity, there are also significant differences between the *S. cerevisiae* and the lager brewer yeast genome. Thus the absolute values for transcript levels can not be obtained from such data. As brewery yeast strains exhibit polyploidity it is possible that some genes are highly expressed due to multiple copies of certain genes. On the other hand, very low expression levels might indicate partial or complete gene deletion. Despite the inability to distinguish between expressions from the two divergent subgenomes, this study provides significant insight into the complexity of regulations for the response to multiple stresses in the course of high gravity beer fermentations. Such information is of great importance for optimization of the design and control of the brewing process (James et al., 2002). To improve the understanding of the brewing process, it is important that in-depth studies is performed with one of the most commonly used industrial lager

beer yeast strains Weihenstephan 34/70 and its native fermentation environment- brewing wort. In conjunction with other "omics" techniques, genome wide transcriptome studies revealed information about the changes necessary for further improvement of the brewing yeast fermentation efficiency.

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References

Affymetrix (2007) Gene chips expression analysis technical manual, P/N 702232 Rev.2

Affymetrix: Affymetrix GeneChip Expression Analysis Technical Manual. Affymetrix, Santa Clara, CA. In.; 2000.

Benjamini Y, Hochberg Y: Controlling the False Discovery Rate: a practical and powerful approach to multiple testing. (1995) J R Statist Soc B, 57(1):289-300.

Blieck L, Toye G, Dumortier F, Verstrepen K, Delvaux F, Thevelein J, Van Dijck P (2007) Isolation and characterization of brewer´s yeast variants with improved fermentation performance under high-gravity conditions. App Environ Microbiol 73:815-824

Bolstad BM, Irizarry RA, Astrand M, Speed TP (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics, 19(2):185-193.

Bond U, Neal C, Donnelly D, James T.C. (2004) Aneuploidity and copy number breakpoints in the genome of lager yeasts mapped by microarray hybridization. Curr. Genet. 45:360-370.

Bose S, Dutko JA, Zitomer RS (2005) Genetic factors that regulate the attenuation of the stress response in yeast. Genetics 169:1215-1226.

Boy-Marcotte E, Perrot M, Bussereau F, Boucherie H, Jacquet M (1998) Msn2p and Msn4p, control a large number of genes induced at the diauxic transition wich are repressed by cyclic AMP in Saccharomyces cerevisiae. J. Bacteriol. 180:1044-1052.

Brosnan MP, Donnelly D, James TC and Bond U (2000) The stress response is repressed during fermentation in brewery strains of yeast J. Appl. Microbiol. 88:746-755.

Causton HC, Ren B, Koh SS, Harbison CT, Kanin E, Jennings EG, Lee TI, True HL, Lander ES, Young RA (2001) Remodeling of yeast genome expression in response to environmental changes. Mol. Biol. Cell 12:323-337.

Gancedo J (1998) Yeast carbon catabolite repression. Microb. Mol. Biol. Rev. 62:334-361.

Gasch AP and Werner-Washburne M (2002) The genomics of yeast responses to environmental stress and starvation. Funct Integr Genom 2:181-192.

Gautier L, Cope L, Bolstad BM, Irizarry RA (2004) Affy--analysis of Affymetrix GeneChip data at the probe level. Bioinformatics 20(3):307-315.

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J *et al* (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 5(10):R80.

Gibson B., Lawrence S., Boulton C., Box W., Graham N., Linforth R. And Smart K. (2008). The oxidative stress response of a lager brewing yeast strain during industrial propagation and fermentation. FEMS Yeast Research, 8(4), 574-585.

Gibson BR, Lawrence SJ, Leclaire JPR, Powell CD, Smart KA. (2007) Yeast responses to stress associated with industrial brewery handling. FEMS Microbiol Rev 31:535-569.

Grotkjaer T, Winther O, Regenberg B, Nielsen J, Hansen LK (2006) Robust multi-scale clustering of large DNA microarray datasets with the consensus algorithm. Bioinformatics 22:58-67.

Higgins VJ, Beckhouse AG, Oliver AD, Rogers PJ, Dawes IW (2003) Yeast genome-wide expression analysis identifies a strong ergosterol and oxidative stress response during the initial stages of an industrial lager fermentation. Appl. Environ. Microbiol. 69:4777-4787.

Hofman-Bang J (1999) Nitrogen catabolite repression in *Saccharomyces cerevisiae*. Mol. Biotech. 12: 35-73.

Hohmann S (2002) Osmotic stress signaling and osmoadaptation in yeast. Microbiol. Mol. Biol. Rev. 66:300-372.

Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP (2003) Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res. 31(4):e15.

James TC, Campbell S, Donnelly D and Bond U (2003) Transcription profile of brewery yeast under fermentation conditions. J. Appl. Microbiol. 94:432-448.

James TC, Campbell SG, Bond UM (2002) Comparative analysis of global gene expression in lager and laboratory yeast strains grown in wort Proc. IEEE 90:1887-1899.

Kellis M, Patterson N, Endrizzi M (2003) Sequencing and comparison of yeast species to identify genes and regulatory elements. Nature. 423:241-254.

Kodama Y., Kielland-Brandt M. C. and Hansen J. in P. Sunnerhagen and J. Piškur (eds.), Comparative Genomics, Springer-Verlag, Berlin Heidelberg, 2005, pp. 145-164, DOI 10.1007/b106370.

Marchler G, Schüller C, Ada G, Ruis H (1993) A Saccharomyces cerevisiae UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. EMBO J 12:1997-2003.

Martínez-Pastor, MT, Marchler G, Schüller C, Marchler-Bauer A, Ruis H, Estruch F (1996) The Saccharomyces serevisiae zink finger proteins Msn2p and Msn4p are required for transcritonal induction throught the stress response elements (STRE). EMBO J. 15:2227-2235.

Nookaew I, Jewett MC, Meechai A, Thammarongtham C, Laoteng K, Cheevadhanarak S, Nielsen J, Bhumiratana S. (2008) The genome-scale metabolic model iIN800 of Saccharomyces serevisiae and its validation: a scaffold to query lipid metabolism. BMC. Syst. Biol. 2: 71-doi:10.1186/1752-0509-2-71

Olesen K, Felding T, Gjermansen C and Hansen J (2002) The dynamics of the Saccharomyces carlsbergensis brewing yeast transcriptome during a production-scale lager beer fermentation. FEMS Yeast Res. 2:563-573.

Oliveira A.P., Patil K.R. and Nielsen J. (2008) Architecture of transcriptional regulatory circuit is knitted over the topology of bio-molecular interaction networks. BMC Syst. Biol. 2: 17-.

Otero JM, Panagiotou G, Olsson L. (2007) Adv. Biochem. Engin/ Biotechnol, 108, , pp. 1-40, DOI 10.1007/10_2007_071.

Patil KR, Nielsen J. Uncovering transcriptional regulation of metabolism by using metabolic network topology. (2005) Proc Natl Acad Sci USA, 102:2685-2689.

Pavlidis (2003) Using ANOVA for gene selection from microarray studies of the nervous system. 31: 282-289.

Pedreño Y, Gimeno-Alcañiz JV, Matallana E, Argüelles J-C (2002) Response to oxidative stress caused by H_2O_2 in Saccharomyces cerevisiae deficient in trehalase genes. Arch. Microbiol. 177:494-499.

Piddocke MP, Kreisz S, Heldt-Hansen H. P., Nielsen K. F., Olsson L (2009) Physiological characterization of brewer´s yeast in high gravity beer fermentations with glucose or maltose syrups as adjuncts. Appl. Microbiol. Biotechnol. online published 3th of April, DOI 10.1007/s00253-009-1930.

Pizarro FJ, Jewett MC, Nielsen J and Agosin E (2008) Growth temperature exerts differential physiological and transcriptional responces in laboratory and wine strains of *Saccharomyces cerevisiae*. Appl. Environm. Microbiol. 74:6358-6368.

Schüller C, Brewster JL, Alexander MR, Gustin MC, Ruis H (1994) The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the Saccharomyces cerevisiae CTT1 gene. EMBO J. 13, 4382-4389.

Shaw SP, Wingfield J, Dorsey MJ and Ma J (1996) Indentifying a species-specific region o9f yeast TFIIB in vivo. Mol. Cell Biol. 16:3651-3657.

Simola M, Hänningen A-L, Stranius S-M, Makarow M (2000) Trehalose is required for comformational repair of heat denaturated proteins in the yeast endoplasmic reticulum but not for maintenance of membrane traffic functions after severe heat stress. Mol. Microbiol. 37:42-53.

Singer MA, Lindquist S (1998) Multiple effects of trehalose on protein folding in vitro and in vivo. Mol. Cell 1:639-648.

Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol., 3:Article 3.

Treger JM, Schmitt AP, Simon JR and McEntee K (1998) Transcriptonal factor mutations reveal regulatory complexities of heat shock and newly identified stress genes in *Saccharomyces serevisiae*. J. Biol. Chem. 273:26875-26879.

Winderickx J, de Winde JH, Crauwels M, Hino A, Hohmann S, Van DP, Thevelein JM. (1996) Regulation of genes encoding subunits of the trehalose synthase complex in *Saccharomyces cerevisiae*: novel variations of STRE-mediated transcription control? Mol. Gen. Genet. 252:470-482.

6

Using metabolome analysis to study the effect of high gravity and ethanol tolerance during brewing

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This chapter represents the technical report of the work

Key words: High gravity, brewer´s yeast, metabolome, ethanol tolerance

Abstract

In the present study, the physiological behavior of lager beer yeast strains with different ethanol tolerance was investigated in the conditions of average (14 ºP) and high gravity (21 ºP) achieved with either glucose or maltose syrup supplementation. The ethanol tolerant strain AJL3126 and the less ethanol tolerant strain AJL2252 were physiologically characterized in comparison to the reference Weihenstephan 34/70. In general, the two strains followed similar fermentation profile compared to strain Weihenstephan 34/70 at the respective wort type and studied gravity. The exception was the poorer fermentation performance of the less ethanol tolerant AJL2252 strain characterized by the higher amount of residual fermentable sugars and lower ethanol yield, in 21 °P glucose syrup supplemented fermentations. In addition, intra- and extracellular metabolome analysis from the stationary phase samples and flavor compounds of the final beer also showed distinct separation for these fermentations from the rest of the studied conditions. Most of the intracellular and extracellular amino acids and TCA cycle intermediates were present in higher concentrations, possibly indicative of restricted growth and leakage of cellular content. Similar effect was observed for metabolome samples from the stationary phase of strain Weihenstephan 34/70 cultivated at 24 °P, regardless of the type of sugar syrup used as adjunct.

Introduction

Metabolites are the end products of cellular regulatory processes and they play a very important role in connecting many different pathways that operate within a living cell. They are defined as low molecular weight compounds, not genetically encoded, produced and modified by the living organisms (Jewett et al., 2006). With the introduction of "-omics" in the system biology world, the word "metabolome" was first used to refer to the entire ensemble of all low-molecular-mass compounds present in and derived from a given living organism (Oliver et al., 1998). Thus, the levels of metabolites can be regarded as the ultimate response of an organism to genetic alterations or environmental influences (Fiehn, 2002). However, the level of the metabolites are determined by the concentration and the properties of the enzymes, and is, therefore, a complex function of many different regulatory processes inside the cell e.g. regulation of transcription and translation, regulation of protein–protein interactions and allosteric regulation of enzymes through their interaction with metabolites. One drawback of global metabolome analysis is that it is highly complicated since it includes all small molecules in the studied biological system. More than 50,000 metabolites have been identified from biological sources and many metabolites are still unknown. Moreover, metabolites are very heterogeneous, have different types of structures, functional groups and physicochemical properties and concentrations varying from mM to less than nM (Villas-Bôas et al, 2004). Nevertheless, the characterisation of the metabolic phenotypes (metabolome), under specific set of conditions can be used to link these phenotypes to their corresponding genotypes integrated or not with gene expression and protein patterns (Villas-Bôas et al, 2005a). Metabolome analysis can, for example, be used: to fingerprint different recombinant strains, permitting rapid phenotypic characterization and to map metabolism in a given strain during fermentation, hereby gaining insight into yeast physiology.

In the case of high-gravity wort fermentations, the ability of yeast to withstand high concentrations of ethanol is an influential factor in strain selection (Briggs et al., 2004; Gibson, 2007). Ethanol tolerate is usually considered to have genetic basis. There is no precise definition of ethanol tolerance and the detailed mechanisms behind it are still not fully known. Despite that ethanol tolerance is commonly strain dependent (Gibson, 2007), it is possible that intermediates of ethanol formation or other products of fermentation exert the deleterious effects on yeast. As wort is highly

complex, its fermentation by brewer´s yeast generates a large number of secondary metabolites which contribute to a different extend to the beer flavour. Thus, the desired concentrations of flavour-active metabolites in yeast are mainly influenced by the choice of yeast strain and wort composition. Gas chromatography (GC) coupled to MS has been extensively used in metabolome analysis because of its high separation efficiency that can resolve very complex biological mixtures. However, the limitation of GC-MS is that the samples must be volatile to be separated on the GC column. Methyl chloroformate (MCF) is a method based on an alkylation reaction using methyl chloroformate (Villas-Bôas, 2005b) and enable simultaneous separation, detection and quantification of more than 70 different amino and non-amino organic acids in a single GC-MS analysis. The main classes to which the flavour compounds in beer belong are aliphatic alcohols, aldehydes, organic and fatty acids and esters of alcohols and fatty acids. All of them are formed as by-products of the metabolism of sugars and amino acids. Previously, wort amino acids have been classified in three groups based on the relative contribution of their corresponding α-ketoacid analogues to the development of balanced spectrum of flavor compounds (Jones and Pierce, 1970). The concentration and type of amino acids present in the wort is of great importance for the brewer´s yeast metabolism and contributes substantially for the flavour composition of the final beer. Furthermore, many of the yeast-derived flavour compounds are excreted by yeast during fermentation. However, some are intracellular metabolites that are released in the beer either as a result of autolysis or via excretion due to stress.

In the present study, the physiological behavior of lager beer yeast strains with different ethanol tolerance was investigated. Metabolome analysis from the early exponential and from the stationary phase of the studied fermentations was carried out, by GC-MS and using MCF derivatization procedure, to reveal the metabolic response for the well ethanol tolerant strain AJL3126, the less ethanol tolerant AJL2252 and the reference Weihenstephan 34/70. The strains were cultivated at average (14°P) and high gravity (21°P) fermentation conditions, achieved with either glucose or maltose syrup supplementation. In addition, the metabolome samples for strain Weihenstephan 34/70 were compared to metabolome samples from previously characterized fermentations at 24 °P.

Materials and methods

Wort

All-malt wort with a gravity of 14 °P and pH=5.2 (purchased from Alectia A/S, Denmark), was used for all fermentations. The wort contained 90% carbohydrates of which the fermentable carbohydrates consisted of 4.4 % fructose, 12.5 % glucose, 66.5 % maltose and 16.7 % maltotriose (w/v). The wort also contained non-fermentable carbon sources such as dextrins and β-glucan. $ZnSO_4.7H_2O$, was added to a concentration of 0.1 ppm Zn. For adjusting the wort to higher gravities - 21° and 24° Plato, respectively, highly fermentable syrups - Clearsweet® 95% Refined Liquid Dextrose Corn Syrup (95.5 % glucose, 2,5 % maltose, 1 % maltotriose, 1 % higher saccharides, present in % dry basis (w/w)) and Satin Sweet® 65 % High Maltose Corn Syrup (70 % maltose, 18 % maltotriose, 2 % glucose, 9 % higher saccharides, present in % dry basis) were used as adjuncts. Both syrups were kindly provided by Cargill Nordic A/S. Prior to inoculation, the wort was oxygenated with air until it reached 100 % saturation. The resulting sugar composition of the different fermentation media used in this study, as measured by HPLC analysis and the used fermentation setup are described in detail in Chapter 3.

Wort density

Wort density and methylene blue staining were measured as previously described (Chapter 3).

Strains

In the present study, three industrial lager beer yeast strains were used. The two strains- AJL3126 and AJL2252 were kindly provided from Alectia A/S collection (former Alfred Jørgensen Laboratories). According to the strain description, AJL3126 is a highly flocculent, and attenuative yeast, which produces a slightly aromatic (estery) and very clean and dry beer. It performs well in high gravity brewing (16-18 °Plato) and in cylindroconical vessels. Strain AJL2252 is moderately flocculent, highly attenuative yeast, which produces an aromatic beer with slight sulphur notes. This yeast is not ethanol tolerant above 14 °Plato and it can not produce a good lager beer at higher temperature (<23°C). The third strain, Weihenstephan 34/70 is commonly used lager beer yeast

strain, purchased from Hefebank Weihenstephan, Freising, Germany. The strains were maintained as a frozen stock culture in 40% (v/v) glycerol.

Fermentation conditions

For the pre-cultures, the yeast from the stock culture was propagated on YPD plates at 30 °C for four days. A single yeast colony was transferred to 20 ml of 14 °P wort in a sterile 50 ml Falcon tube and incubated at 25 °C in a rotary shaker at 150 rpm. After 48 hours, the preculture was transferred to a shake flask with 375 ml of fresh wort and incubated for 72 hours.

All fermentations were performed in 2.2 liter bioreactor (Biostat B5; Braun Biotech International, Melsungen, Germany) with a working volume of 1.5 liter. Dissolved oxygen was monitored with an autoclavable polarographic oxygen electrode. The fermentors were integrated with Braun Biotech Multi-Fermenter Control System (MFCS) for data acquisition. Silicone based antifoam agent FD20P in concentration of 0.1 ml/L (Basildon Chemicals, England) with a food gradient quality was used in the fermentations. The reactors were inoculated with a volume of pre-culture, corresponding to 1×10^7 cells/ml. During the cultivation the temperature was maintained at 14 °C and the stirring was set to 90 rpm. Prior to sampling the stirring was increased to 300 rpm for 2 min. The higher stirring allowed better mixing and homogenization of the media and ensured representative sampling. The pH was recorded on-line, but not controlled. After the fermentation was completed, the whole fermentation broth was transferred to a sterile vessel and stored for 14 days at 0°C, for further maturation. Detailed physiological characteristics of the cultivations performed and the wort compositions at 14, 21 and 24 °P fermentations has been described previously (Chapter 3).

Calculations of specific growth rate and yield coefficients

The specific growth rate was determined as the slope from the linear function of the ln (logarithmic function with base 10) of the cell number (cells/ml) and the fermentation time (h) during the exponential growth phase. The yield coefficients were determined as the slope from the linear regression on the corresponding pairs of substrate (total saccharides) and product concentration (glycerol and ethanol).

Free amino nitrogen (FAN) analysis

The levels of free amino nitrogen (FAN) of the unfermented worts and from the last day of the beer fermentation were determined using the ninhydrin method at 570 nm (EBC 1998). Glycine was used as a standard.

HPLC analysis

A Dionex Summit HPLC system (Synnyvale, CA) was used for analysis of sugars and metabolites from the extracellular medium as previously described (Chapter 3).

Headspace analysis of esters and higher alcohols

GC-FID analysis of the esters and higher alcohols of the final beer samples was performed using a Perkin-Elmer Autosystem XL gas chromatograph equipped with automatic HS40 XL headspace autosampler. Detailed description of the method is present in Chapter 3.

Sampling

Samples for analysis of sugars and alcohols were collected every 24 hours throughout the fermentation. For measuring the free amino nitrogen content, samples were collected from the first and the final day of the primary fermentation. For the all of the above analyses, 2-10 ml of fermentation samples were withdrawn from the fermentor, immediately filtered through a Cameo 0.20 µm pore size acetate/glass filters (Sartorius AG, Germany) and stored at -20 °C prior to analysis.

Metabolome analysis

After withdrawal from the fermentor, the samples were rapidly quenched into 20 ml precooled (-40 °C) 72 % methanol (de Koning and van Dam, 1992). Cells were centrifuged at 10 000 x g for 20 minutes at -20 °C to separate them from the quenching solution. Further on, the intracellular metabolites were extracted using chloroform: methanol: 3 mM Pipes buffer (pH=7) extraction (Villa-Boas et al., 2005). Following extraction, the samples were lyophilized using a Christ-Alpha 1-4 freeze dryer.

Following lyophilisation, the samples were resuspended in 200 µl of 1% (w/v) NaOH solution and derivatised using the methodology by Villas-Bôas et al., (2003). Peak detection was conducted with

AMDIS (NIST, Gaithersburg, MD) using default parameters. In order to decrease the matrix effect in the extracellular samples containing a high concentration of maltodextrins (sugars), the samples were resuspended in 2 ml of 1% (w/v) NaOH solution. As external standards, amino acid standards (Sigma) at two different levels were used. As internal standards 20 mM of EDTA and 30 mM of chlorophenylalanine were used. Samples were normalised by the amount of intracellular standards and by the cell number and expressed in peak area. GC-MS analysis was performed with a Hewlett-Packard system HP 6890 gas chromatograph coupled to a HP 5973 quadrupole mass selective detector (EI) operated at 70 eV. For the analyses, column J&W1701 column with size (30m ×250 µm × 0.15 µm) was used (Folsom, CA). The used GC-MS program has previously been described by Panagiotou et al., 2007. Peak detection was conducted with AMDIS (NIST, Gaithersburg, MD) using default parameters.

Data analysis

Three-ways ANOVA was used to compare the effect of increased gravity and fermentation phase for strain Weihenstephan 34/70 with those effects for two other strains- the well ethanol tolerant AJL 3126 and the less ethanol tolerant AJL 2252. For this analysis, three factors: gravity, strain and growth phase were used. The factor gravity was present at two levels: average (14 °P) and high (21 °P), the factor growth phase was also present at two levels (exponential and stationary) and the factor strain was present at three levels (Weihenstephan 34/70, AJL 3126 and AJL 2252). For both ANOVA analyses, the p-values were corrected for multiple testing by applying the False Discovery Rate (FDR) methodology described by Benjamini and Hochberg (Benjamini and Hochberg, 1995) and the significant metabolites were selected by imposing a cut-off value of 0.05.

Additionally, for strain Weihenstephan 34/70, two-ways ANOVA was used in order to identify significantly changed metabolites with respect to the effect of increased gravity and growth phase. The factor “gravity” was present at three levels: average (14), high (21) and very high (24). The factor “growth phase” was present at two levels- early exponential and stationary. The used model was fitted to study the effect of these two main factors along with their interaction factor (‘wort type*fermentation phase’).

Results

Physiological characterization of strain AJL 3126 and AJL 2252

In the present study, the well ethanol tolerant strain AJL 3126 (here in called AJL1) and the less ethanol tolerant strain AJL 2252 (here in called AJL2) were physiologically characterized at average gravity- 14 °P and at high gravity- 21 °P, achieved with the addition of either glucose (Gl) or maltose (M) syrup to the basic wort. In addition, their physiological characteristics were compared to those of the previously characterized strain Weihenstephan 34/70 under the same fermentation conditions (Chapter 3). In general, the fermentation profiles for both AJL 3126 and AJL 2252 strains were similar to the fermentation profile of strain Weihenstephan 34/70 at the respective wort type and studied gravity. Exception was the poorer fermentation performance of the less ethanol tolerant AJL 2252 strain, compared to the fermentation performance of the other two strains, in 21 °P glucose syrup supplemented fermentations (**Figure 6-1**; **Table 6-1**). This poorer fermentation performance resulted in slightly lower ethanol yield during exponential growth phase - 0.45 (g/g) and very high amount of residual fermentable sugars (up to 45 g/L at the end of the fermentations), resulting in significantly lower overall ethanol yield based on total consumed sugars. In comparison, the fermentations in 21 Gl °P wort for strain Weihenstephan 34/70 and AJL 3126 resulted in an ethanol yield of 0.48 (g/g) and 0.49 (g/g), respectively (**Table 6-1**) and 18 g/L and 10 g/L of residual fermentable sugars, respectively. Another interesting observation was the observed strain difference in the glycerol yield. Despite the similar growth rate among the three wort types, while for strain AJL 3126 highest glycerol yield was observed for the 14 °Plato fermentations, for strains Weihenstephan 34/70 and AJL 2252, highest glycerol yield was observed for the 21 °Plato glucose syrup supplemented fermentations.

The flavour profiles from the final beer for the 14 ºP fermentations was similar among the three strains. The flavour profiles of the final beer produced with strain AJL 3126 and Weihenstephan 34/70 at the 21 °Plato were very similar, the fermentations with strain AJL 2252 resulted in different flavor profile of the final beer, both for the glucose and maltose supplemented fermentations (**Figure 6-2**). The most pronounced difference was higher concentration for propanol and lower concentrations for the rest of the studied flavor compounds (**Figure 6-2A** and **6-2B**). In addition, while for strain AJL3126 and AJL2252 the 21 ºP fermentations had similar profile for the glucose and maltose syrup supplemented fermentations, for strain AJL 2252, bigger differences among the two conditions were observed.

Table 6-1. Growth characteristics of the studied fermentations.

Strain	Wort °P	Specific growth rate (h^{-1})	Glycerol yield (g glycerol/g sugars)	Ethanol yield (g ethanol/g sugars)
AJL1	**14 P**	0.05	0.022	0.43
	21Gl	0.070	0.005	0.49
	21M	0.059	0.004	0.48
AJL2	**14 P**	0.04	0.008	0.48
	21Gl	0.045	0.019	0.47
	21M	0.056	0.011	0.46
W34/70	**14 P**	0.067	0.021	0.45
	21Gl	0.05	0.026	0.48
	21M	0.064	0.018	0.49

[a] *Values are the average of two independent batch cultivations perform in duplicate (n=2). Standard deviations were determined to be lower then 5% of the average value.*

[b] *Yields are calculated as g of products (ethanol, glycerol) produced per g of total sugars consumed from the exponential phase.*

A. B.

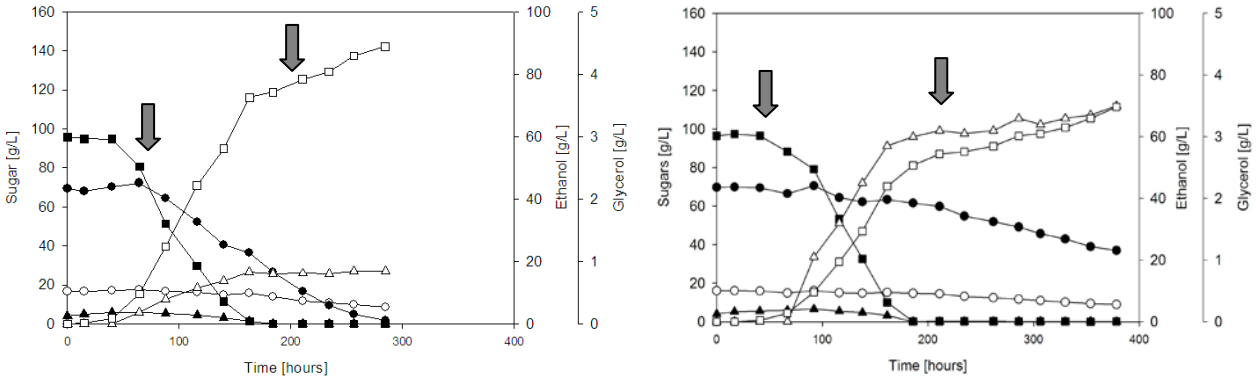

Figure 6-1. Fermentations with glucose syrup supplementation: *A. Strain AJL 3126; B. Strain AJL 2252; The arrows in the figures correspond to the sampling points for metabolome analysis of the studied fermentations. The symbols in the graphs represent the concentrations of: " ■ " glucose, "▲"fructose, " ● " maltose, " ○ " maltotriose, " □ "ethanol," △ "glycerol.The arrows indicate the sampling points for the metabolome analysis.*

A. B.

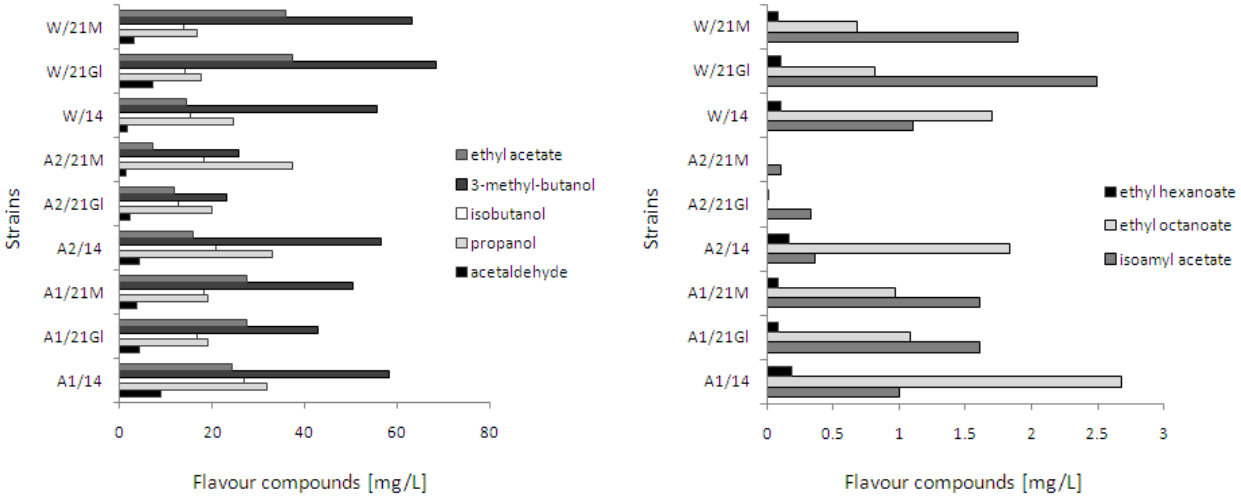

Figure 6-2. Flavour profile of the studied fermentations: *A. Flavour compounds present in higher concentrations; B. Flavour compounds present in lower concentrations. The following abbreviations represent: W- strain Weinhenstephan 34/70; A2- strain AJL 2252; A1- strain AJL 3126. The numbers 14 and 21 refer to the respective gravity of the wort. M- represents addition of maltose rich syrup and Gl- represents addition of glucose rich syrup.*

In order to investigate the effect of the increased gravity on the fermentation performance of the less ethanol tolerant strain AJL 2252, compared to the well ethanol tolerant AJL 3126 and the reference strain Weihenstephan 34/70 (Chapter 3), samples for intracellular metabolome analysis from the early exponential phase and both intra and extracellular metabolome samples from the stationary phase of the fermentations at 14 and 21 °Plato (achieved with the addition of either glucose or maltose syrups to the basic wort) were investigated (**Figure 6-1**).

Metabolome analysis

In the current study, the principle of alkylation reaction using methyl chloroformate (Villas-Bôas, 2005) was used to enable simultaneous separation, detection and quantification of both intracellular and extracellular metabolites, belonging to the groups of amino acids and non-amino organic acids as well as their derivatives. From the approximately 600 metabolites included in the genome-wide metabolic reconstruction for yeast (Förster et al., 2003), 40% are amines, amino acids and organic acids. Out of those, the used in-house method, has a MS library consisting of 75 metabolites playing a major role in the central carbon metabolism and amino acid biosynthesis.

Metabolome analysis of strains AJL 3126, AJL 2252 and Weihenstephan 34/70

In total, for the three strains comparison, 45 intracellular and 22 extracellular metabolites from the early exponential phase and 37 intracellular and 19 extracellular metabolites from the stationary phase of the studied fermentations at 14, 21Gl and 21M °Plato were determined.

ANOVA analyses of the metabolome samples from the early exponential phase investigating the strain factor revealed the amino acids: asparagine, glycine, lysine, N-acetyl-L-glutamate, cystathionine and tyrosine as significantly changed. The same metabolites with the addition of tyrosine were also found to be significantly changed when investigating the effect of type of sugar syrup used in the fermentations and for the interaction effect of gravity and strain used (**Table 6-2**).

Table 6-2. Significantly changed metabolites from the early exponential phase of strain AJL 3126, AJL 2252 and Weihenstephan 34/70 based on ANOVA analyses considering the factors type of sugar used to increase the gravity, strain and the interaction effect of both.

Metabolites		p-value		
Short abbreviation	**Full name**	**Sugar effect**	**Strain effect**	**Interaction effect of sugar and strain**
ASN	Asparagine	0.001	0.000	0.001
GLY	Glycine	0.037	0.014	0.037
LYS	Lysine	0.037	0.014	0.037
NAGLU	N-acetyl-L-glutamate	0.061	0.019	0.061
LLCT	Cystathionine	0.061	0.019	0.061
TYR	Tyrosine	0.149		0.149

Bigger differences among the metabolome samples for the three strains where observed from the stationary phase of the studied fermentations (**Table 6-3**).

Table 6-3. Significantly changed metabolites from the stationary phase of strain AJL 3126, AJL 2252 and Weihenstephan 34/70 based on ANOVA analyses considering the factors type of sugar syrup used to increase the gravity, strain and the interaction effect of both.

	Full metabolites names	**Effect of gravity and sugar type**		**Effect of strain**		**Interaction effect of gravity and sugar type used**	
		Metabolites (short abbreviation)	p-value	Metabolites (short abbreviation)	p-value	Metabolites (short abbreviation)	p-value
Metabolites common for all three factors	Citramalic acid	CITMex	9.84E-47	CITMex	2.32E-46	CITMex	8.19E-47
	Isocitric acid	ICIex	9.84E-47	ICIex	2.32E-46	ICIex	8.19E-47
	Trans-4-hydroxyproline	HPRO	0.001	HPRO	0.001	HPRO	0.000
	O-acetyl-L-Serine	ASER	0.002	ASER	0.005	ASER	0.002
	Triptophan	TRP	0.002	TRP	0.005	TRP	0.002
	Cystathionine	LLCT	0.002	LLCT	0.005	LLCT	0.002
	Valine	VAL	0.010	VAL	0.018	VAL	0.009
	2-aminobutyric acid	2-ABA	0.010	2-ABA	0.028	2-ABA	0.011
	Lactic acid	LACex	0.028	LACex	0.009	LACex	0.036
	Citric acid	CITex	0.028	CITex	0.009	CITex	0.020
Metabolites common for the effects of gravity and strain used	Ethyl octanoate	ETHOCT	0.002	ETHOCT	0.013		
Metabolites common for the effect of gravity and interaction effect	Ethyl hexanoate	ETHHEX	0.008			ETHHEX	0.036
Metabolites common for the effect of strain and interaction effect	Ethyl acetate			ETHAC	0.018	ETHAC	0.038
Metabolites unique for the specific effect	Asparagine	ASN	0.046				
	Methyl-butanol			METBUT	0.040		
	Glutamine					GLUN	0.042
	Citric acid			CITex	0.009		
	Alanine					ALA	0.044

The abbreviation "ex" in the short name for some of the metabolites refers to the extracellular metabolites.

A closer look of the concentration for the significant –intra/-extracellular metabolites from the stationary phase and the flavour compounds from the final beer from the three strains comparison revealed that citramalic and isocitric acid were present only in the 14 ºP fermentations for strain Weihenstephan 34/70; ethyl hexanoate and ethyl octanoate were nearly absent from the final beer produced from 21 °Plato glucose syrup supplemented fermentations with the less ethanol tolerant AJL 2252, while at the same time extracellular citric acid and lactic acid for example are present in significantly higher concentrations compared to the rest of the studied conditions.

To explore the data and reduce its dimensionality, principal component analysis (PCA) was applied to the normalized metabolome data. Three principal components where observed comprising 65% of the variance for the three strains comparison.

Accounting for all of the determined metabolites from the early exponential phase samples of the three strains comparison, the 21 ºPlato samples for strain AJL 2252 both with glucose and maltose syrup supplementation and the 21 ºPlato glucose supplemented fermentations for strain Weihenstephan 34/70 showed clear separation from the rest of the studied conditions, both for PC1 versus PC2 and for PC1 versus PC3, while the samples from the rest of the studied conditions grouped together (**Figure 6-3**). Full list of the determined metabolites from the three strains comparsion used for the ANOVA analyses and their relative concentration after normalization with internal standard and biomass concentration is present respectively in **Table 6-1S** and **Table 6-2S**, supplementary information.

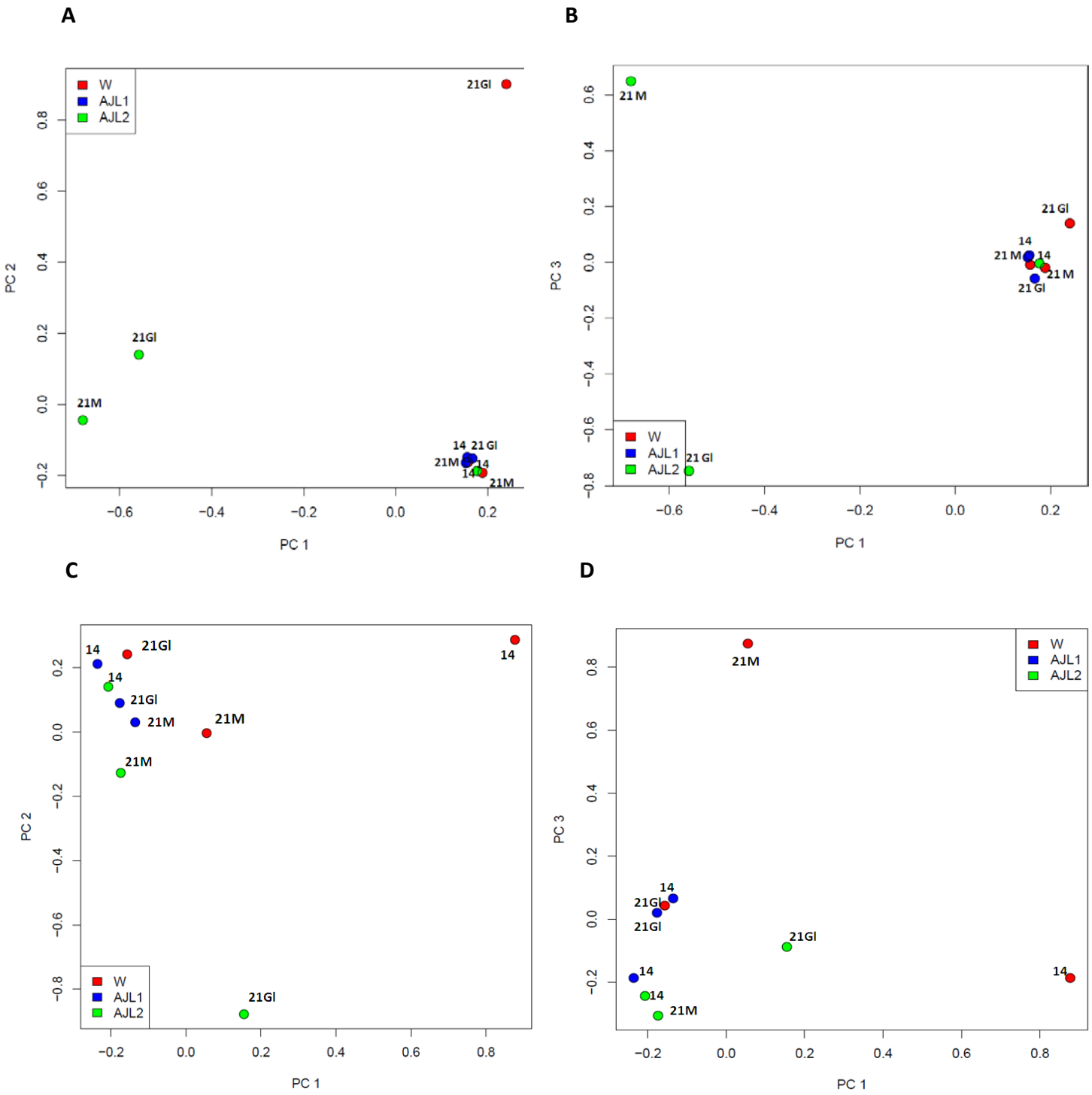

Figure 6-3. PCA projection in the first three PCA dimensions of samples from three strains comparison from fermentations at 14, 21Gl and 21M Plato fermentations. *Plot A shows the segregation of PC1 versus PC2 and plot B shows the segregation of PC1 versus PC3 for intra- and extracellular metabolome samples from the early exponential phase of 14, 21Gl and 21M °Plato fermentations for strain AJL 3126, strain AJL 2252 and strain Weihentephan 34/70. Plot C and D shows the segregation of PC1 versus PC2 and Plot D shows the segregation for PC1 versus PC3 for intracellular metabolome samples and flavor compounds from the stationary phase of the studied fermentations. The numbers 14 and 21 refer to the respective gravity of the wort. M- represents addition of maltose rich syrup and Gl- represents addition of glucose rich syrup.*

Broader separation among the principal components was observed for the stationary phase samples. However, again, the most distinct separation was observed for samples from strain AJL 2252 from 21 ºP glucose syrup supplemented wort. Distinct separation was also observed for 14 ºP wort for strain Weihenstephan 34/70.

Metabolome analysis from the stationary phase of strain Weihenstephan 34/70 cultivated at average (14 °P), high (21 °P) and very high (24 °P) gravity

In total, for the metabolome samples from the stationary phase of strain Weihenstephan 34/70 grown at 14, 21Gl, 21M, 24Gl and 24M °P fermentations, 43 intracellular metabolites were determined to be present in at least one of the studied conditions. Ten of the detected metabolites, in addition to the flavour component ethyl acetate, were significantly changed among the studied conditions. The significant intracellular metabolites and flavour compounds from the two-way ANOVA analyses are presented in **Table 6-4**. Clearly, the ANOVA analysis among the studied conditions revealed the influence of increase in the gravity on essential for the brewer´s yeast fermentation and flavours formation amino acids such as valine, leucine, methionine, phenylalanine, tryptophan, tyrosine and threonine (**Table 6-4**). The normalised relative concentrations of the significantly changed metabolites were significantly higher for the 24 °Plato fermentations, compared to the lower gravity fermentations.

PCA analysis (**Figure 6-4**) including the detected intracellular metabolites from the stationary phase together with the determined flavour compounds from the final beer (Chapter 3), revealed clear separation of the samples from 24 °Plato fermentations from the rest of the fermentations regardless whether glucose or maltose syrups were used as adjunct to increase the gravity. Three principal components where observed comprising up to 75% of the variance.

Additions of sugar syrups to increase the gravity resulted in dilution of the nitrogenous content in the wort. Both the present metabolome analysis from the stationary phase samples and earlier physiological characterisation and characterisation of the amino acids profile (Chapter 3) and global transcriptome analysis from those fermentations (Chapter 5) confirm that restricted growth and incomplete fermentation is mostly associated with 24 °Plato fermentations.

Table 6-4. Significant intracellular metabolites (ANOVA analyses) from the stationary phase of beer fermentations with strain Weihenstephan 34/70 grown at 14, 21 and 24 ºPlato fermentations. *The numbers 14 and 21 refer to the respective gravity of the wort. M- represents addition of maltose rich syrup and Gl- represents addition of glucose rich syrup.*

Metabolites	**Wort type**					
Full name	**14 P**	**21Gl**	**21M**	**24Gl**	**24M**	**p-value**
Valine	77.0	0.7	4.4	1643.1	1634.3	0.001
Leucine	15.1	0.6	2.1	1956.5	1939.4	0.001
Methionine	0.0	0.0	0.0	3.7	3.8	0.002
Phenylalanine	119.8	0.1	0.5	2452.6	2377.6	0.005
Trypthopan	57.5	0.1	0.5	200.3	207.7	0.006
Tyrosine	91.3	0.4	1.4	297.8	282.8	0.011
Proline	63.9	15.0	8.3	5149.6	5626.4	0.024
Ethyl acetate	14.5	37.4	36.0	39.4	37.8	0.033
(3S)-3-Methyl-2-oxopentanoic acid	0.0	0.0	0.0	0.7	0.9	0.036
Threonine	35.1	0.0	0.9	135.6	154.0	0.036
Cumarine	0.0	0.0	0.0	2.9	2.5	0.036

The metabolites are present in relative concentrations, after normalization with the biomass concentrations at the sampling point and internal standard.

A. B.

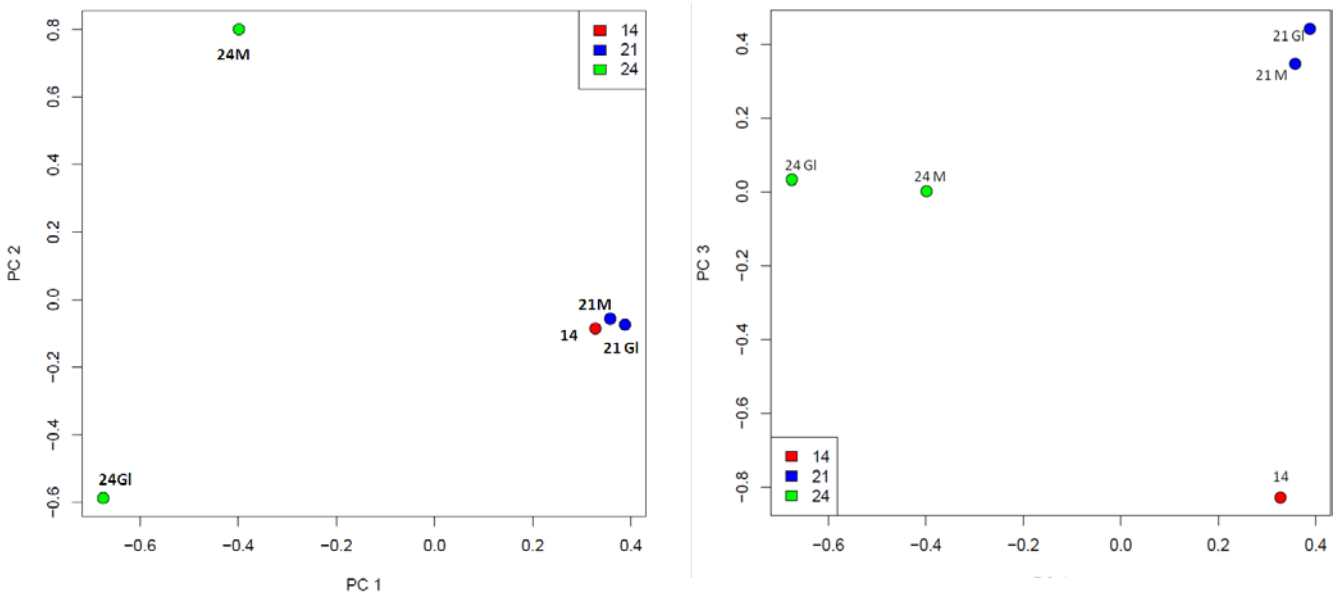

Figure 6-4. PCA projection in the first three PCA dimensions of samples for intracellular metabolome and flavor compounds from the stationary phase of strain Weinhenstephan 34/70. *Plot A shows the segregation of PC1 versus PC2 and plot B shows the segregation of PC1 versus PC3.*

Full list of the determined metabolites used for the ANOVA analyses for the stationary phase samples with strain Weihenstephan 34/70 at 14, 21 and 24 °P and their relative concentration after normalization with internal standard and biomass concentration is present respectively in **Table 6-3S**, supplementary information.

Discussion

Metabolome footprinting have previously been used to differentiate ale from lager brewing yeast strains (Pope et al., 2007). The present study also reveals that intra- and extracellular metabolome profiling in addition to physiological and flavour profile characterisation can be used to distinguish among individual lager beer yeast strains with different ethanol tolerance.

An interesting observation for the physiological characterization of the three studied strains with different ethanol tolerance was the difference in glycerol yield. In the case of strains Weihenstephan 34/70 and AJL 2252, glycerol accumulation was coupled to the cell growth or accumulated to higher concentrations when glucose syrup was used as adjunct to increase the gravity. The accumulation of glycerol to higher concentrations in the glucose rich wort can also be explained with the effect of the initiation of the high-osmolarity glycerol (HOG) pathway, designed to promote survival of the organisms during period of physiological stress. The induction of the HOG pathway ultimately results in stimulating the hyperproduction and hyperaccumulation of glycerol as a compatible solute in order to balance the external and internal osmolarities (Gibson et al., 2007). On the other hand, as it is known that strain AJL 3126 is well ethanol tolerant and it has obviously been selected and optimized for growth at high gravity and to withstand production of as high as possible ethanol concentrations, this strain had much lower glycerol yield at 21 °P fermentations.

It has previously been shown that ethanol concentrations as low as 5 % (w/v) reduce water availability sufficiently to have metabolic consequences. While at average gravity (10-14 °P) brewing yeast strains are exposed to ethanol concentrations typically in the range of 3-6% (v/v), in high gravity brewing, ethanol concentrations can reach up to 10% v/v (Briggs et al., 2004). In a response to ethanol exposure, as is the case of stationary phase of high gravity beer fermentation, brewer´s yeast undergo morphological changes such as cell surface invaginations and cell shrinkage (Pratt-Marshall et al., 2003). In a response to this stress, yeast synthesizes and accumulates compounds that protect the structure and function of the cellular components. Yeast cells accumulate polyols such as glycerol, amino acids such as proline, and/or the disaccharide trehalose (Hallsworth, 1997). In the present study, intracellular proline accumulation to higher concentrations was observed in the 24 °P fermentations (**Table 6-4**). Some strains, as in the case of strain AJL 3126 can better withstand the deleterious effects of ethanol compared to others. In order to generate high ethanol concentrations during fermentation, higher initial sugar concentration is necessary,

consequently strains capable of withstanding the ethanol stress should also be capable of maintaining its growth also at the conditions of low water activity. Despite that ethanol tolerance is commonly strain dependent, it is possible that intermediates of ethanol formation or other products of fermentation exert the deleterious effects on yeast.

At the conditions of ethanol toxicity, the major effect of ethanol is on the cell membrane and other intracellular membranes. Thus, under conditions of ethanol toxicity, leakage of cellular components, abolishment of membrane proton motive potential, inhibition of transport systems and alterations in membrane structure and fluidity has been observed (Briggs et al., 2004; Gibson, 2007). Exposure of yeast to very high ethanol concentrations results in inhibition of growth, which was also observed in the present study, in the case of strain AJL 2252. Similar effect was also observed for the better ethanol tolerant Weihenstephan 34/70 under very high gravity fermentations- 24 ºP. The normalised relative concentrations of the significantly changed metabolites were present in significantly higher concentration for the 24 °Plato fermentations, compared to the lower gravity fermentations. Additions of sugar syrups to increase the gravity resulted in dilution of the nitrogenous content in the wort. Both the present metabolome analysis from the stationary phase samples and earlier physiological characterisation and characterisation of the amino acids profile (Chapter 3) and global transcriptome analysis from those fermentations (Chapter 5) confirm that restricted growth and incomplete fermentation is mostly associated with 24 °Plato fermentations.

Organic acids are largely derived from the incomplete TCA cycle that occurs during anaerobic repressed growth of yeast or from the catabolism of amino acids (Briggs et al., 2004). In the amino acid biosynthesis of yeast, valine is derived from pyruvate, leucine from acetyl-CoA, proline from α-ketoglutarate, tryptophan from phosphoenol-pyruvate, phenylalanine and tyrosine from erythrose-4-phosphate. As most of those amino acids are derived from TCA cycle intermediates, their intracellular accumulation towards the end of very high gravity fermentations is indicative of incomplete TCA cycle associated with restricted growth. Possibly, this can be explained by the combined effect of observed stuck fermentation at this gravity, resulted in restricted growth and by the eventual effect of ethanol toxicity, due to the high ethanol concentrations at the end of very high gravity fermentations.

The differences in ethanol tolerance and type of sugar syrup used as adjunct to increase the gravity also resulted in differences in the flavour profile of the final beer. The determined flavour profile resulting from the AJL 2252 fermentations at 21 ºPlato showed significant differences compared to the rest of the fermentations both for the glucose and maltose supplemented fermentations.

The resulting flavour profile was possibly also predetermined by the observed higher relative concentration of intracellular amino acids from the early exponential phase of the maltose syrup supplemented fermentations and from the stationary phase of the glucose syrup supplemented fermentations at 21 °P (**Table 6-1S** and **Table 6-2S**, supplementary information).

In conclusion, intra- and extracellular metabolome analysis in addition to physiological, and flavor profile characterization can distinct between different lager beer strains and metabolome analysis can be used as a tool for monitoring industrial fermentations and as such predict the outcome of the flavour profile of the final beer. Such applications can possibly be used as well in further fermentation optimization strategies for different brewer´s yeast strains.

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References

Briggs DE, Boulton CA, Brookes PA, Stevens R (2004) Brewing: Science and practice. Woodhead, Cambridge, UK.

de Koning, W., van Dam K. **(**1992) A method for the determination of changes of glycolytic metabolites in yeast on on a subsecond time scale using extraction at neutral pH K. Anal. Biochem. 204:118−23.

Fiehn O. (2002). Metabolomics- the link between genotypes and phenotypes. Plant. Mol. Biol. 48, 155-171.

Gibson B.R., Lawrence S. J., Leclaire J. P. R., Powell C. D., Smart K. A. (2007) Yeast responses to stresses associated with industrial brewery handling. FEMS Microbiol. Rev. 31: 535-569.

Hallsworth J.E. (1998). Ethanol-induced water stress in yeast. J. Ferm. Bioeng.85:125-137.

Jewett, M.C., Hofmann G. and Nielsen J. (2006) Fungal metabolite analysis in genomics and phenomics. Curr. Opin. Biotechnol. 17:191-197.

Oliver S.G., Winson M.K., Kell D.B. and Baqanz F. (1998) Systematic functional analysis of the yeast genome. Trends Biotechnol. 16:373-378.

Panagiotou G, Kouskoumvekaki I, Jónsdóttir S. O, Olsson L. (2007) Monitoring novel metabolic pathways using metabolomics and machine learning: induction of phosphoketolase pathway in *Aspergillus nidulans* cultivations. Metabolomics 3:503-516.

Pierce JS, Jones, M (1970) Proc. 25th Cong Eur. Brew. Conv., Interlaken, p. 151.

Pope G., MacKenzie D., Defernez M., Aroso M., Fuller L., Mellon F., Dunn W., Brown M., Goodacre R., Kell D., Marvin M., Louis E. and Roberts I. (2007) Yeast 24, 667-679.

Pratt P, Bryce J, Stewart G (2003) The effect of osmotic pressure and ethanol on yeast viability and morphology. J. Inst. Brew. 109: 213-228

Villas-Bôas S. G., Rasmussen S, Lane G.A. (2005a). Metabolomics or metabolite profiles? Trends Biotechnol. 23, 385.

Villas-Bôas S.G. (2005) Mass spectrometry in metabolome analysis. Mass Spectr. Rev.24: 613 – 646.

Villas-Bôas S.G., Moxley J., Akesson M., Stephanopoulos G., Nielsen J. (2005b) High-throughput metabolic state analysis: the missing link in integrated functional genomics of yeasts. Biochem J. 388:669-677.

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Effect of organic and inorganic nitrogen source additions in high gravity beer fermentations on the brewer´s yeast metabolism

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Abstract

High gravity brewing is a process using wort with a higher sugar concentration than normal. Addition of sugar syrups to the wort is a popular technique to increase gravity but results in decreased levels of free amino nitrogen in the wort leading to nitrogen limitation during fermentation. With the goal of understanding how to provide more available nitrogen in high gravity beer fermentations, we studied the effects of adding nitrogen sources with an organic (urea) or inorganic (ammonium sulphate) nature on 21 °Plato beer fermentations using the lager beer brewer's yeast strain Weihenstephan 34/70. We also investigated the possibility for utilizing unavailable nitrogen from the wort, such as longer chain peptides, by adding proteases as a supplement to the wort. The fermentations with nitrogen source supplementation had a longer exponential growth phase compared to the control fermentations. Among the studied nitrogen sources, we found that the fermentations with Flavourzyme supplementation resulted in the highest specific growth rate and ethanol yield, highest specific ethanol and glycerol productivities and lowest amount of residual sugars.

Introduction

High gravity brewing is the process of using wort with a higher sugar concentration than normally used. Because of the high ethanol content produced during fermentation the resulting product is diluted to the desired alcohol content. A popular approach for increasing sugar concentration in wort is adding sugar syrups as adjuncts to wort with average gravity (usually in the range of 12-14 °Plato). However, as sugar syrups do not normally contain nitrogen, their addition to the basic wort will dilute the free amino nitrogen (FAN) content present in the media. On the other hand, to ensure optimal specific growth rates and thus successful brewer´s yeast fermentation, it is a requirement that wort at higher gravity has higher free amino nitrogen content compared to wort at lower gravity. Under anaerobic conditions, while FAN requirements for wort of 12 °Plato are in the range of 140-150 mg/L, wort with >18 °Plato requires FAN of 280 mg/L (O´Conor-Cox, 1991). To a certain extent, FAN requirements are also strain specific (Quain and Boulton, 2006).

Nitrogen is an essential nutrient for yeast growth and it is used primarily by the cells to form amino acids and nucleic acids for its anabolism. *Saccharomyces cerevisiae* has the ability to grow on ammonium, urea and most amino acids as a sole nitrogen source (Henschke and Jiranek, 1994). In *S. cerevisiae*, 12 constitutive and four nitrogen repressible amino acid carriers have been identified (Horak, 1986). The transporters can be of high or low affinity type, their uptake is active and involves proton symport. *S. cerevisiae* is able to utilise a wide variety of nitrogenous compounds such as amino acids, urea and GABA (γ-aminobutyrate). The general amino acid permease (GAP1) with broad specificity and high capacity is responsible for transport of these compounds against the concentration gradient. In *S. cerevisiae*, there are also a number of transporters specific for only one amino acid or for small groups of amino acids. The nitrogenous compounds can either be directly incorporated into the protein synthesis pathways, or be degraded to one of the end products, ammonium and glutamate (Hofman-Bang, 1999). These two compounds, together with glutamine, play an essential role in central nitrogen metabolism (Beltran, 2005) as they link both catabolism and anabolism. Through their interconnection, ammonium and glutamate together with glutamine act as the ultimate precursor of all nitrogen biosynthetic reactions.

Wort contains a complex mixture of nitrogen sources and yeasts possess transport systems mediating the uptake of both organic and inorganic nitrogen sources. The major sources of nitrogenous compounds in wort are amino acids and ammonium ions. The catabolism of amino

acids provides precursors for other nitrogenous containing cellular constituents such as purines and pyrimidines.

The main group of flavour metabolites are higher alcohols, aldehydes, organic acids, fatty acids and esters of alcohols and fatty acids. They are all formed as by-products of the metabolism of sugars and amino acids. Thus, the amino acids metabolism and their profile during the course of beer fermentation will greatly influence the flavour profile of the final beer. As in the case with carbon source utilization, uptake of nitrogenous nutrients is an ordered process. Presence in the medium of readily utilisable assimilable nitrogen sources represses the synthesis of transporter proteins and catabolic enzymes of other, less readily utilised nitrogen sources. This phenomenon is known as the nitrogen catabolite repression (Wiame et al., 1985).

Among the inorganic nitrogen sources, ammonium salts are the only ones, which can be utilized by yeast. Due to its cost effectiveness and metabolic efficiency, ammonium is widely used as a nitrogen source in industrial fermentations. Ammonium (and also glutamate) are efficient nitrogen sources since they directly enter the intracellular pool of biosynthetic precursors.

Although ammonia is usually utilised by yeasts in preference to the organic nitrogen sources, during fermentation, brewer´s yeast utilises several amino acids before utilising ammonium ions. Since brewer´s yeast prefers asparagine, glutamine and glutamate, these nitrogen sources will be used first in addition to ammonia. In the absence of those primary compounds, the yeast will use secondary nitrogen sources, such as other amino acids, smaller peptides and amides (Batistote et al., 2006).

Urea is widely used as an inexpensive nitrogen source and most yeast strains can also utilise urea. If the concentration of urea in the media exceeds 0.5 mM, it enters the yeast cells by facilitated diffusion. When the concentration is below this limit, urea is taken up by active transport (Walker, 1998). Urea is often used in industrial fermentations, but its use is not recommended as a nutritional supplement in fermentations for spirit beverage production since residual urea and ethanol can react during distillation and form ethyl carbamate, a compound considered to be carcinogenic (Walker, 1998).

The utilization of amino acids is more complex than the utilization of the primary sources, since their metabolism require the synthesis of specific catabolic enzymes and permeases (Batistote et al. 2006). Besides amino acids, short peptides, containing less than five amino acid residues can also be taken up by yeast, but not as readily as free amino acids. *S. cerevisiae* strains do not produce

exogenous proteases, and since they are not capable of transporting bigger peptides inside the cells, they are not able to utilise them.

To approach the industrial challenge of providing more available nitrogen in high gravity beer fermentations, we tested the effects of the addition of nitrogen sources of organic and inorganic nature on the metabolism of lager beer strain Weihenstephan 34/70. In addition, with the goal of making nitrogen sources already present in the wort available to the yeast, such as longer chain peptides, we investigated the effect of addition of proteases as a supplement to 21 °Plato high gravity beer fermentations.

Materials and methods

Strain

The flocculent bottom fermenting industrial lager beer yeast strain Weihenstephan 34/70 (Hefebank Weihenstephan, Freising, Germany) was used in this study. The strain was maintained as a frozen stock culture in 40 % (v/v) glycerol.

Wort

All-malt wort with a gravity of 14 °P and pH=5.2, (purchased from Alectia, Denmark), was used for all fermentations. The wort contained 90% carbohydrates of which the fermentable carbohydrates consisted of 4.4 % fructose, 12.5 % glucose, 66.5 % maltose and 16.7 % maltotriose (w/v). The wort also contained non-fermentable carbon sources such as dextrins and β-glucan. $ZnSO_4.7H_2O$, was added to a concentration of 0.1 ppm Zn. For adjusting the wort to higher gravities- 21° and 24° Plato, respectively, highly fermentable syrups-Clearsweet® 95% Refined Liquid Dextrose Corn Syrup (95.5 % glucose, 2,5 % maltose, 1 % maltotriose, 1 % higher saccharides, present in % dry basis (w/w) and Satin Sweet® 65 % High Maltose Corn Syrup (70 % maltose, 18 % maltotriose, 9 % higher saccharides, 2 % glucose, present in % dry basis) were used as adjuncts. Both syrups were kindly provided by Cargill Nordic A/S. Prior to inoculation, the wort was oxygenated with air until it reached 100 % saturation.

Nitrogen source supplementation

In order to select the nitrogen sources giving the most desired fermentation performance in terms of specific growth rate, lag phase until ethanol production started, sugar uptake rate and flavor and aroma profile, we performed initial screening experiments with the selected nitrogen sources at several different concentrations. Summary of the type of the nitrogen sources and their concentrations used for the screening experiments is presented in **Table 7-1**.

Table 7-1. Experimental setup for the screening experiment.

Nitrogen source	Nitrogen source concentration
Urea	10 mM
	50 mM
	100 mM
Ammonium sulphate	10 mM
	50 mM
	100 mM
Flavourzyme	30 ppm
	60 ppm
Thermolysin	50 ppm
	200 ppm
Thermolysin + Amino peptidase II	22.5 ppm Thermolysin + 5 ppm Aminopeptidase II
	200 ppm Thermolysin + 25 ppm Aminopeptidase II

[a] *The fermentations were run in duplicates.*

[b] *All supplementations were tested at 21 ˚P both with glucose and maltose syrup supplementation.*

External supplementation

It is known that among the inorganic nitrogen sources, *Saccharomyces* yeasts cannot utilize nitrate or nitrite, but readily assimilable ammonium ions. In the present study, ammonium sulphate was used as an inorganic nitrogen source, while urea was used as an organic nitrogen source. Both sources were tested at three different concentrations (**Table 7-1**).

Enzymatically generated nitrogen source

The nitrogen content in wort accounts for 4-5 % of the total dissolved solids. Fractionation of the soluble nitrogen in wort has shown that 58 % of the total soluble nitrogen is in a form of free amino acids and peptides, 22 % of it is polypeptides and 20 % is proteins. Thus, up to 42 % of potential nitrogenous source remains unused in the course of beer fermentation.

In an attempt to target the remaining unused proteins and peptides in the media, the effect of the addition of one single enzyme and two enzyme mixtures with peptidase activities was investigated with the goal of elucidating the effect of their addition on the wort fermentability and brewer´s yeast metabolism. One combination used was the enzyme Thermolysin (commercially available enzyme, Sigma) and Aminopeptidase II (laboratory trial enzyme, Novozymes A/S). Thermolysin is a thermostable neutral metalloproteinase enzyme produced by the gram-positive bacteria *Bacillus thermoproteolyticus.* It has an endopeptidase activity, with pH optimum at 8.0 and optimal activity at 70 ^{o}C. Aminopeptidase II is a laboratory trial enzyme with exopeptidase activity. Its optimal performance is achieved at pH= 9.7, at 70 °C.

The other enzyme mixture used was the commercially available enzyme Flavourzyme (Novozymes A/S), which is a multicomponent enzyme with both endo- and exo- peptidase activities. Previously, Flavourzyme has been commercially used to boost the nitrogen supplementation in various food fermentations. The optimal performance for this enzyme mixture is with pH in the range of 5.0- 7.0 at 50 °C.

The effect of Flavourzyme, Thermolysin, and the combination of Thermolysin with Amino peptidase II were investigated at two different concentrations (**Table 7-1**).

Fermentation conditions

For the pre-cultures, yeast from the stock culture was propagated on YPD plates at 30 °C for four days. A single yeast colony was transferred to 20 ml of 14 °P wort in a sterile 50 ml Falcon tube and incubated at 25 °C in a rotary shaker at 150 rpm. After 48 hours, the preculture was transferred to a 500 ml shake flask with 375 ml of fresh wort and incubated for 72 hours. Both shake flasks and bioreactors were inoculated with 1×10^7 cells/ml. The shake flask cultivations were performed at 14 ^{o}C, on a rotory shaker with a speed of 120 rounds per minute (rpm).

The selected fermentation conditions for further characterisation in Braun Biostat Fermentors are presented in Table 2. All bioreactor fermentations were performed in 2.2 liter bioreactors (Biostat B5; Braun Biotech International, Melsungen, Germany) with a working volume of 1.5 liters. Dissolved oxygen was monitored with an autoclavable polarographic oxygen electrode. The fermentors were integrated with the Braun Biotech Multi-Fermenter Control System (MFCS) for data acquisition. Silicone based antifoam agent FD20P at a concentration of 0.1 ml/L (Basildon Chemicals, England) with a food grade quality was also used in the fermentations. The reactors were inoculated with a volume of pre-culture, corresponding to 1×10^7 cells/ml. During the cultivation the temperature was maintained at 14 °C and the stirring was set to 90 rpm. Prior to sampling the stirring was increased to 300 rpm for 2 min. The higher stirring allowed better mixing and homogenization of the media and ensured representative sampling. The pH was recorded on-line, but was not controlled. When the pH and the gravity remained constant for two consecutive days, the fermentation was considered completed. After the fermentation was completed, the whole fermentation broth was transferred to a sterile vessel and stored for 14 days at 0°C for further maturation.

Table 7-2. Overview of the different fermentation conditions.

	21 °Plato Maltose	21 °Plato Glucose
Control		
Urea	**10 mM**	**10 mM**
Flavourzymes	**60 ppm**	**60 ppm**
Thermolysin + Aminopeptidase II	**200 ppm + 22.5 ppm**	**200 ppm + 22.5 ppm**

All fermentations were run in duplicates

Sampling

Shake flask cultivations

Due to the observed flocculation, prior to sampling, the shake flasks were placed on a magnet stirrer until the content was homogenous. The sample was removed using a syringe and needle through the rubber membrane placed on the arm of the flask. The rubber membrane was rinsed with ethanol before and after sampling.

Bioreactor fermentations

Samples for analysis of sugars, alcohols and amino acids were collected every 24 hours throughout the fermentation. For measuring the free amino nitrogen content, samples were collected from the first and the final day of the primary fermentation. For all of the above analyses, 2-10 ml of fermentation samples were withdrawn from the fermentor, immediately filtered through a Cameo 0.20 µm pore size acetate/glass filters (Sartorius AG, Germany) and stored at -20 °C prior to analysis. Samples for flavor and aroma compound analyses were collected after 14 days of maturation, filtered and stored at -20 °C prior to analysis.

Methylene Blue staining

Viability tests were done using the methylene blue staining method according to EBC Analytica (Hjortshöj et al., 1992) and the viable yeast cells were counted in a Bűrker-Tűrk cell counting chamber.

HPLC analysis

Carbohydrates and alcohols

A Dionex Summit HPLC system (Synnyvale, CA) was used for analysis of sugars and metabolites from the extracellular medium. All metabolites were detected refractometrically (Waters 410 Differential Refractometer Detector, Millipore Corp., Milford, MA) after separation on an Aminex HPX-87H column (Biorad, Hercules, CA) at a temperature of 60 ^{o}C using 5 mM H_2SO_4 as eluent. To allow the separation of the sugars with different degrees of polymerization, two Aminex columns were mounted in serial with isocratic elution at 0.40 ml/min. External standards of maltotriose (DP3), maltose (DP2), glucose (DP1), fructose (DP1), glycerol and ethanol were used for external quantification at 6 different levels.

Amino acids

The 20 essential individual amino acids were quantified on a Dionex Summit HPLC system after derivatization in an alkaline buffer with o-phthalaldehyde (OPA) (primary amino acids) and 9-flourenylmethyl chloroformate (FMOC) (secondary amino acids) (Herbert et al., 2001).

The amino acid derivatives were separated on a 150 x 4.60 mm, 3 µm, Gemini C_{18} column (Phenomenex, Torrance, CA). Detection was performed using a RF200 fluorescent detector, using 288 nm as excitation wavelength and 305 nm as emission wavelength. Separation was performed using a linear binary gradient 1.0 ml/min of A (20 mM thriethylamine adjusted to pH 7.5 Using acetic acid) and B (45 % acetonitrile, 45 % methanol and 10 % water). The gradient started with 12% B for 12 min and was then increased linearly to 40 % B for 38 min and further increased to 61 % for 9 min, after which it increased to 100 % in 1 min, where it was maintained for 2 min before reverting to the starting conditions in 4 min. Norvalin with concentration of 0.25 mM, diluted in 0.1 M HCl was used as an internal standard, and Sigma Amino Acid Standard (AAS18), containing seventeen out of the twenty analysed amino acids, additionally supplemented with asparagine, glutamine and tryptophan were used for calibration at six different levels.

Free amino nitrogen (FAN) analysis

The levels of free amino nitrogen (FAN) of the unfermented worts and from the last day of the beer fermentation were determined using the ninhydrin method at 570 nm (Analytica-EBC, 1998; Lie, 1973). Glycine was used as a standard.

Urea determination

The levels of urea from the fermentations with urea supplementation was determined spectrophotometrically, using urea/ammonia enzymatic kit (R- Biopharm, Germany).

Headspace analysis of esters and higher alcohols

GC-FID analysis for six of the main esters and higher alcohols of the final beer samples was performed using a Perkin-Elmer Autosystem XL gas chromatograph equipped with automatic HS40 XL headspace autosampler. Samples of 5.0 ml were transferred to 20-ml auto sampler vials and capped with butyl-PFTE seals. Samples were thermostated for 30 min at 60 °C, pressurized with 3.5 bar helium, and transferred to the GC trough a 0.25 mm ID deactivated fused silica transfer line held at 90 °C. Injection to the analytical column DB-5 (60 m, 0.25 mm, ID 1.0 µm film, J&W Scientific) was done in split mode at a ratio of 1:10 for 1.2 min. The initial oven temperature was set at 35 ºC for 2 min, followed by an increase with 6 ºC/min until the temperature reached 200 ºC. Flame ionization temperatures was 250 ºC, and helium was used as the carrier gas at a constant flow of 35 cm/s. Perkin-Elmer Turbochrom Navigator software was used for instrument control. N-butanol was used as an internal standard, and calibrated against all six studied compounds: acetaldehyde, ethyl acetate, isoamyl acetate, ethyl hexanoate, ethyl octanoate, propanol, isobutanol, 3-methyl-butanol at six levels.

Calculations of specific growth rate and yield coefficients

The specific growth rate was determined as the slope from the linear function of the ln (logarithmic function with base 10) of the cell number (cells/ml) and the fermentation time (h) during the exponential growth phase. The yield coefficients were determined as the slope from the linear regression on the corresponding pairs of substrate (total saccharides) and product concentration (glycerol and ethanol, respectively).

Results

From an industrial point of view, the desired improvement in high gravity beer fermentation is to achieve a shorter lag phase, a higher ethanol yield and high specific ethanol productivity, and at the same time as low biomass and off-flavour formation as possible.

Screening experiment

Sugar utilization and ethanol formation

In order to investigate the effect on the addition of the different nitrogen sources on the wort fermentability, biomass formation and ethanol yield, screening experiments were carried out. The experiments were done in duplicates, at two or three different concentrations for each of the studied nitrogen sources (**Table 7-1**). Thus, the purpose of the shake flask screening experiments was to select the best performing cultivation conditions based on the above criteria.

The results from the screening experiment in high gravity wort using glucose and maltose syrups as adjuncts, respectively, are presented in **Table 7-3**. In general, the nitrogen source supplementation at the lowest concentrations resulted in better performance or equally well as at higher concentrations. Both for the glucose and maltose syrup supplemented fermentations, the best performing conditions in terms of high ethanol yield, specific growth rate and respectively specific ethanol productivity were the fermentations with Flavourzyme supplementation.

Table 7-3. Ethanol yield and specific growth rate of the high gravity control and nitrogen supplemented fermentations.

	21 °P Gl		21 °P M	
	Ethanol yield (g/g)	**Specific growth rate (h^{-1})**	**Ethanol yield (g/g)**	**Specific growth rate (h^{-1})**
Control	0.42	0.050	0.46	0.064
Urea 10 mM	0.41	0.024	0.45	0.068
Urea 50 mM	0.43	0.032	0.44	0.071
Urea 100 mM	0.40	0.074	0.41	0.072
$(NH_4)_2$ SO_4 10 mM	0.40	0.072	0.47	0.072
$(NH_4)_2$ SO_4 50 mM	0.33	0.070	0.41	0.046
$(NH_4)_2$ SO_4 100 mM	0.40	0.071	0.44	0.032
Flavourzymes 30 ppm	0.40	0.071	0.46	0.072
Flavourzymes 60 ppm	0.44	0.072	0.46	0.071
Thermolysin 50 ppm	0.32	0.074	0.34	0.021
Thermolysin 200 ppm	0.33	0.075	0.33	0.022
Thermolysin 22,5 ppm / APII 5ppm	0.36	0.072	0.33	0.039
Thermolysin 200 ppm / APII 25ppm	0.36	0.075	0.4	0.052

Urea

Both for the glucose and maltose syrup supplemented fermentations, increase in the urea concentration from 10 to 50 and 100 mM resulted in decreased ethanol yield, but higher specific growth rate (h^{-1}) (**Table 7-3**).

Ammonium sulphate

With increase in the ammonium sulphate concentrations from 10 to 50 and 100 mM, both for the glucose and maltose supplemented fermentations, the ethanol yield and the specific growth rate decreased (**Table 7-3**). Among all studied conditions, the fermentations with ammonium sulphate supplementation resulted in the highest amount of residual sugars at the end of the fermentation. The residual sugar observed for the maltose supplemented fermentations, increased from 3.3 g/L for the 10 mM supplementation to 5 g/L for the 100 mM supplementation. While the fermentations with glucose syrup supplementation and 10 mM ammonium sulphate supplementation had 11 g/L of residual sugars, the fermentations with 100 mM supplementation ended with 51.5 g/L of residual sugar. While glucose and fructose were completely consumed, maltose and maltotriose were the main contributors to the residual sugars. In comparison, the amount of residual sugar for the fermentations with other nitrogen sources was in the range from 1.5 to 3.9 g/L, with no significant difference in the level if glucose or maltose syrups were used as adjuncts.

Enzyme supplementation

While for the maltose supplemented fermentations, an increase in the Flavourzyme concentrations from 30 to 60 ppm resulted in ethanol yield of 0.46 g/g and specific growth rate of 0.071-0.072 h^{-1} for both conditions, for the fermentations with glucose syrup supplementation, increase in the Flavourzyme concentrations from 30 to 60 ppm resulted in a similar specific growth rate – from 0.071 h^{-1} and 0.072 h^{-1}, but an increase in the ethanol yield- from 0.40 to 0.44 g/g (**Table 7-3**). Fermentations with Thermolysin supplementation resulted in similar specific growth rates (0.074-0.075 $h^{-1)}$ and ethanol yields (0.32 to 0.34 g/g) under all tested conditions. Supplementation with Aminopeptidase II in addition to Thermolysin resulted in improved fermentation performance in terms of higher ethanol yields and higher specific growth rates compared to the fermentations with only Thermolysin supplementation. Furthermore, for the maltose supplemented fermentations, increase in the concentrations of Thermolysin/Amino peptidase II, resulted in increased ethanol yield and specific growth rate.

Despite the observed differences among the different concentrations of the supplemented nitrogen sources, nitrogen supplementation did not result in higher ethanol yield compared to the control fermentations. Instead, the best performing nitrogen supplemented fermentations resulted in lower

or similar to the controls values for the ethanol yield, but higher specific growth rates, resulting in higher specific ethanol productivity ($g/g/h^{-1}$) (data not shown).

Free amino nitrogen (FAN) and urea determination

The free amino nitrogen value represents the amount of nitrogenous compounds present in the wort - amino acids, ammonia, small peptides and terminal α – amino nitrogen groups of bigger peptides and proteins. While most of these compounds are utilized by brewer´s yeast, the disadvantage of the FAN method is that it also partially measures proline, an amino acid that cannot be utilized by the brewer´s yeast. Urea, as such, can not be measured by this method. Thus, the FAN values from the urea supplemented fermentations do not account for the urea addition.

As high gravity beer fermentations have demand for higher FAN, compared to lower gravity fermentations, FAN value must be sufficiently high to ensure that lack of nitrogenous compounds does not limit the fermentation, but that optimal specific growth rate and complete sugar utilization can be reached. Prior to nitrogen supplementation, the initial concentration of FAN in the cultivation media was in the range of 190-201 mg l^{-1} both for the glucose and for the maltose syrup supplemented fermentations.

As the FAN method determinates the concentration of ammonium ions present in the wort, as expected, for the ammonium sulphate supplemented fermentations, significant increase in the FAN concentrations from the early exponential phase were observed, compared to the rest of the studied conditions. Increase in the initial FAN concentrations of the supplementation with ammonium sulphate also resulted in significant increase of the final FAN value (data not shown). Urea concentrations can not be measured by the FAN method, thus urea addition did not result in increase in the FAN concentrations from the early exponential phase. However, since in the process of urea degradation ammonium is released, increase in the concentrations of urea supplementation resulted in a significant increase of the final FAN values (data not shown).

The FAN values from the screening experiments for the control and protease supplemented fermentations are presented in **Table 7-4**. As proteases supplementation resulted in increase in the liberated FAN, comparison between the control and the studied proteases supplemented

fermentations also revealed higher amount of consumed FAN during the exponential growth phase for the protease supplemented fermentations.

Table 7-4. FAN measurements (in mg l^{-1}) from the screening experiment.

	21 °P Glucose	**21 °P Maltose**
	Consumed FAN (mg l^{-1})	**Consumed FAN (mg l^{-1})**
Control	116	124
Flavourzymes 30 ppm	132	154
Flavourzymes 60 ppm	141	143
Thermolysin 50 ppm	114	77
Thermolysin 200 ppm	134	99
Thermolysin 22,5 ppm / Aminopeptidase II 5ppm	126	96
Thermolysin 200 ppm / Aminopeptidase II 25ppm	133	116

[a] *The control fermentations are without additional nitrogen source supplementation to the basic wort.*

[b] *The values are average of duplicate measurements with standard deviation < 10%.*

[c] *The consumed FAN is expressed as the difference in the FAN values between the early exponential and stationary phase.*

Besides sufficient amount of free amino nitrogen at the beginning of the fermentation, another important criterion for optimal beer fermentation is the amount of residual FAN. High levels of residual peptides and amino acids contribute to high residual FAN values, which in turn result in higher concentration of higher alcohols and might negatively influence the flavour and aroma profile of the final beer (De Rouck et al., 2007). Among the studied conditions, it was observed that the final FAN is lowest for the fermentations with enzyme addition, particularly in the case of Flavourzyme supplementation. The observed FAN values from the end of the beer fermentation

were in the range of 38-50 mg l^{-1} for the Flavourzyme supplemented fermentations and in the range of 51 to 73 mg l^{-1} for Thermolysin and Thermolysin/Aminopeptidase II supplementations.

Based on the screening experiments, the cultivations that gave highest ethanol yield, and lowest residual sugar concentrations, in combination with low residual FAN values at the end of the fermentations, were selected for further characterisation in bioreactor fermentations. Despite the observed high ethanol yield and specific growth rate for the ammonium sulphate supplemented fermentations, high residual sugar concentrations resulted for the glucose syrup supplemented fermentations, and consequently ammonium sulphate was not chosen as a nitrogen supplement for the further experimental trials. The urea supplemented cultivations resulted in higher specific growth rate, but lower ethanol yield compared to the control fermentations and as urea supplementation at 10 mM resulted in the highest ethanol yield and specific growth rate, it was decided to select supplementation with 10 mM for further characterisation in bioreactor fermentations.

Among all the studied conditions, the fermentations with Flavourzyme supplementation resulted in the best fermentation performance both for the glucose and maltose syrup supplementation, resulting in the highest ethanol yield, highest specific growth rate and thus high specific ethanol productivity together with lowest observed FAN values at the end of the fermentations. To ensure that the enzyme was present in sufficient concentration for the bioreactor experiments, it was chosen to use the higher tested concentrations from the screening experiment for the enzymes supplementation.

Both the fermentations with Thermolysin and Thermolysin/Aminopeptidase II supplementations gave the highest specific growth rate (0.072- 0.075 h^{-1}), but lowest observed ethanol yield among the studied conditions. The ethanol yield was in the range of 0.31-0.32 (g/g) for the single Thermolsyin supplementation and 0.33-0.40 (g/g) for the combination of Thermolysin/Aminopeptidase II supplementation. Because of the relatively lower ethanol yield compared to the other studied conditions, and high amount of residual sugar observed at the end of the fermentations, in addition to viscosity problems caused by the increased viscosity of the media, the fermentations with addition of Thermolysin as a single enzyme were not investigated further.

To ensure that both enzymes are present in sufficient concentrations for the bioreactor fermentations, we decided to select the supplementation with Thermolysin/Aminopeptidase II at 200/22.5 ppm.

Bioreactor cultivations

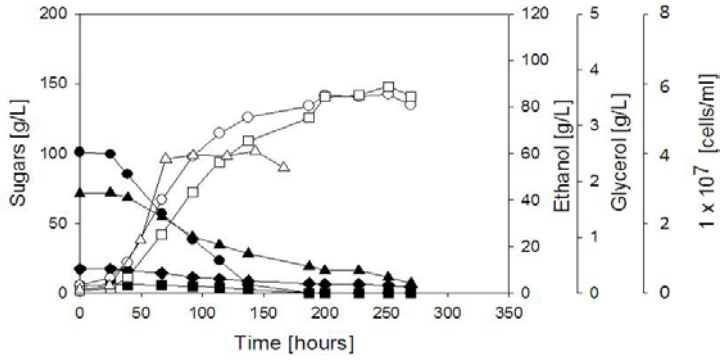
Based on the above observations, the best performing fermentation conditions from the shake flask experiments were selected for further characterisation in bioreactors. The cultivations were characterised in terms of sugars, FAN and amino acid utilisation, ethanol and glycerol formation and flavour and aroma compound characterisation.

Physiological characterisation

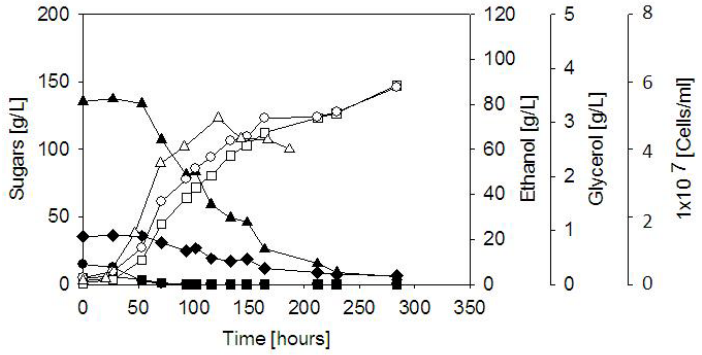
The fermentation profile of the studied fermentations is presented in **Figure 7-1** and **Table 7-5**.

In general, the results from the bioreactor fermentations supported the results from the shake flask experiments. The fermentations with nitrogen source supplementation resulted in longer exponential growth phase and more complete sugar utilization compared to the control fermentations. In all cases, the residual sugars were maltose and maltotriose. While the control fermentations resulted with up to 20 g/L of residual sugars, the fermentations with nitrogen supplementation ended with residual sugars in the range of 3-4 g/L (**Figure 7-1**). The only exception was the fermentations with Thermolysin/Aminopeptidase II with glucose syrup wort supplementation which had up to 15 g/L of residual sugars. However, for the fermentations with urea and particularly the fermentations with Thermolysin/Aminopeptidase II supplementation more complete sugar utilization was associated with longer fermentation time compared to the control and to Flavourzyme supplemented fermentations (**Figure 7-1**).

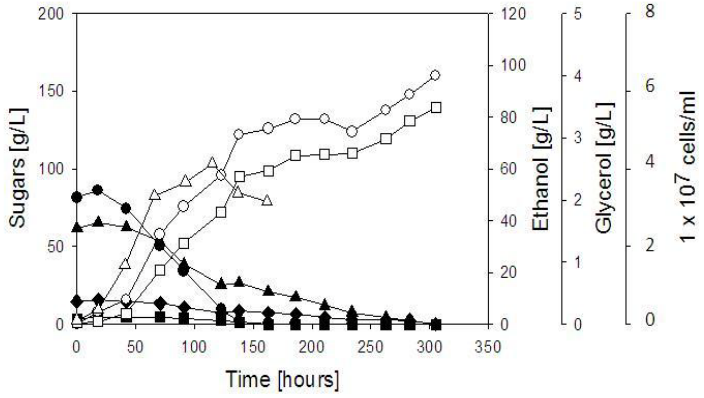
A.


Sugars [g/L]
Ethanol [g/L]
Glycerol [g/L]
1 x 10^7 [cells/ml]
Time [hours]

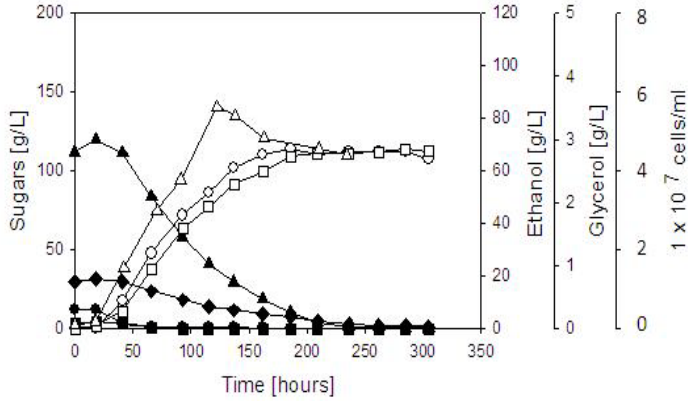
B.


Sugars [g/L]
Ethanol [g/L]
Glycerol [g/L]
1x10^7 [Cells/ml]
Time [hours]

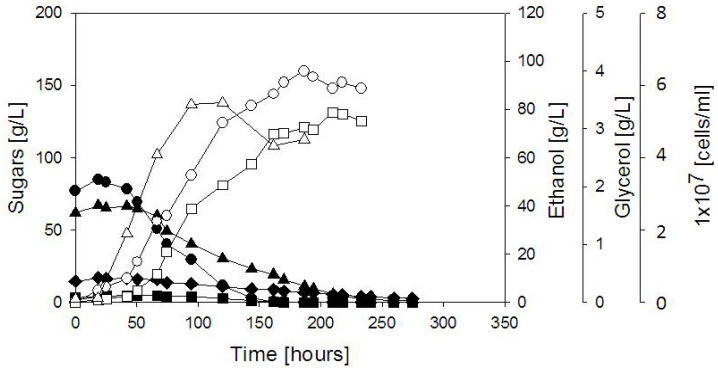
C.


Sugars [g/L]
Ethanol [g/L]
Glycerol [g/L]
1 x 10^7 cells/ml
Time [hours]

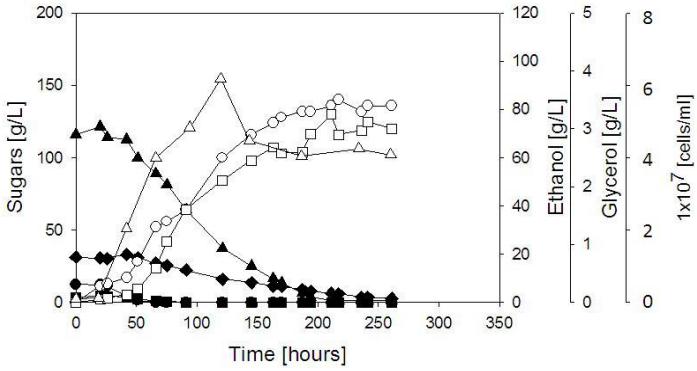
D.


Sugars [g/L]
Ethanol [g/L]
Glycerol [g/L]
1 x 10^7 cells/ml
Time [hours]

E.


Sugars [g/L]
Ethanol [g/L]
Glycerol [g/L]
1x10^7 [cells/ml]
Time [hours]

F.


Sugars [g/L]
Ethanol [g/L]
Glycerol [g/L]
1x10^7 [cells/ml]
Time [hours]

G. **H.**

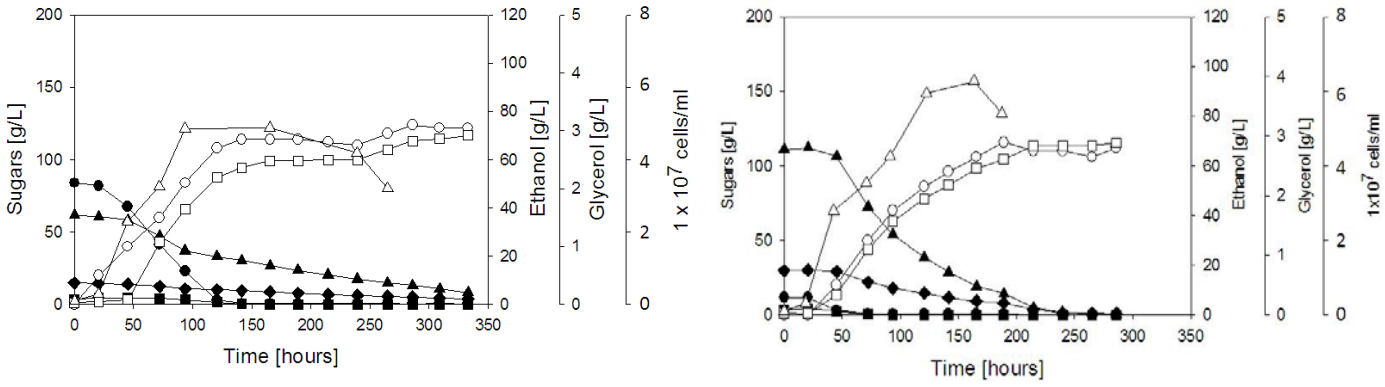


Figure 7-1.Time course during fermentations showing sugar consumption and product formation *A. Control fermentation with glucose syrup supplementation; B. Control fermentation with maltose syrup supplementation; C. Glucose syrup supplemented fermentation with urea addition. D. Maltose syrup supplemented fermentation with urea addition E. Glucose syrup supplemented fermentation with Flavourzyme addition. F. Maltose syrup supplemented fermentation with Flavourzyme addition G. Glucose syrup supplemented fermentation with Thermolysin/Amino peptidase addition. H. Maltose syrup supplemented fermentation with Thermolysin/Amino peptidase addition.*

Among the fermentations with different nitrogen sources, the fermentations with Flavourzyme supplementation resulted in the highest specific growth rate and ethanol yield, lowest amount of residual sugar and high FAN utilization. Furthermore, Flavourzyme addition was the only supplementation that resulted in significantly higher specific growth rate compared to the control fermentations (**Table 7-5**). The fermentations with urea and Thermolysin/Aminopeptidase II supplementation had similar or lower specific growth rate, compared to the control. In general, the fermentations with nitrogen supplementation had similar ethanol yields as the control. The nitrogen supplementation did not result in further increase of the ethanol yield. Among the studied conditions, Flavourzyme was the only exception which showed higher ethanol yield compared to the control for the maltose supplemented fermentations (**Table 7-5**).

For the glucose syrup supplemented fermentations, the highest glycerol yield was observed for the control fermentations, while for the maltose syrup supplemented fermentations the glycerol yield was similar, both for the control and for the nitrogen supplemented fermentations (**Table 7-5**).

Table 7-5. Physiological and growth characteristics of the nitrogen supplemented fermentations.

Wort °P	**21 °P Glucose**				**21 °P Maltose**			
	Control	**Urea**	**Flavourzyme**	**Thermolysin + Amino peptidase II**	**Control**	**Urea**	**Flavourzyme**	**Thermolysin + Amino peptidase II**
Specific growth rate (h^{-1})	**0.050**	**0.052**	**0.074**	**0.041**	**0.064**	**0.047**	**0.074**	**0.042**
Ethanol yield (g/g)	**0.48**	**0.46**	**0.48**	**0.47**	**0.49**	**0.43**	**0.47**	**0.45**
Glycerol yield (g/g)	**0.026**	**0.022**	**0.023**	**0.023**	**0.018**	**0.017**	**0.018**	**0.018**
Initial FAN (mg/L)	**210**	**229**	**257**	**292**	**220**	**236**	**234**	**279**
Final FAN (mg/L)	**55**	**119**	**52**	**59**	**52**	**133**	**60**	**44**

Free amino nitrogen and urea determinations

We measured the free amino nitrogen concentrations at the beginning and at the end of the primary fermentations (**Table 7-5**). Both urea and enzyme supplemented fermentations showed increased FAN values at the beginning of the fermentations. The highest observed initial FAN value was found for the Thermolysin/APII supplemented fermentations, followed by the Flavourzyme supplemented fermentations. Despite the increase in the initial FAN values, both cases of enzyme supplementation resulted in final FAN values similar to the control fermentations. Thus, the additionally liberated FAN amount was also consumed by the brewer´s yeast.

Urea supplementation was the only exception which resulted in high residual FAN values. This effect is possibly a result of two combined events. On one side, urea degradation results in an increase in the ammonium concentrations leading to increased FAN values. On the other hand, urea is one of the most preferred nitrogen sources by yeast and will preferably be utilized compared to many of the amino acids. Determination of urea from the urea supplemented fermentations both for

the glucose and for the maltose supplemented fermentations showed gradual decrease in the urea concentrations for the first hundred fermentation hours (corresponding to the exponential growth phase) where 60-70 % of the supplemented urea was consumed, after which the residual urea concentrations remained constant (data not shown).

Amino acid metabolism

The pattern of the amino acid uptake in the nitrogen supplemented fermentations followed the pattern of the amino acid uptake of the control fermentations. In all cases, first the amino acids from group A (including aspartic acid, glutamic acid, asparagine, glutamine, serine, threonine, lysine, arginine were consumed, followed by consumption of amino acids from group B (valine, methionine, leucine, isoleucine, histidine) and group C (glycine, phenylalanine, tyrosine, tryptophan, alanine).

There was no significant difference in the amount of the free amino acid concentrations measured between the control and the nitrogen supplemented fermentations (data not shown). As beer batch fermentation is a dynamic process in which amino acids are both taken up and released in the media, the actual increase in the amino acid concentrations is difficult to determine. However, in all cases of enzyme supplemented fermentations shorter lag phase prior utilisation of the amino acids was observed compared to the control fermentations. Such effect was not observed for the urea supplemented fermentations. Since urea is the preferred nitrogen source instead of some of the individual amino acids, availability of urea in the fermentation wort slowed down the assimilation for the less preferred amino acids. Thus, longer lag phase prior utilisation of the amino acids of group B and group C from the urea supplemented fermentations was observed (data not shown).

Flavour and aroma profile

Important considerations when investigating the nitrogen supplementation of beer fermentations is its effect on the flavour and aroma profile of the final beer. In the present study, the concentrations of acetaldehyde, two esters- ethyl and isoamyl acetate and three high alcohols - isobutanol, 3-methyl butanol and propanol were measured (**Figure 7-2**).

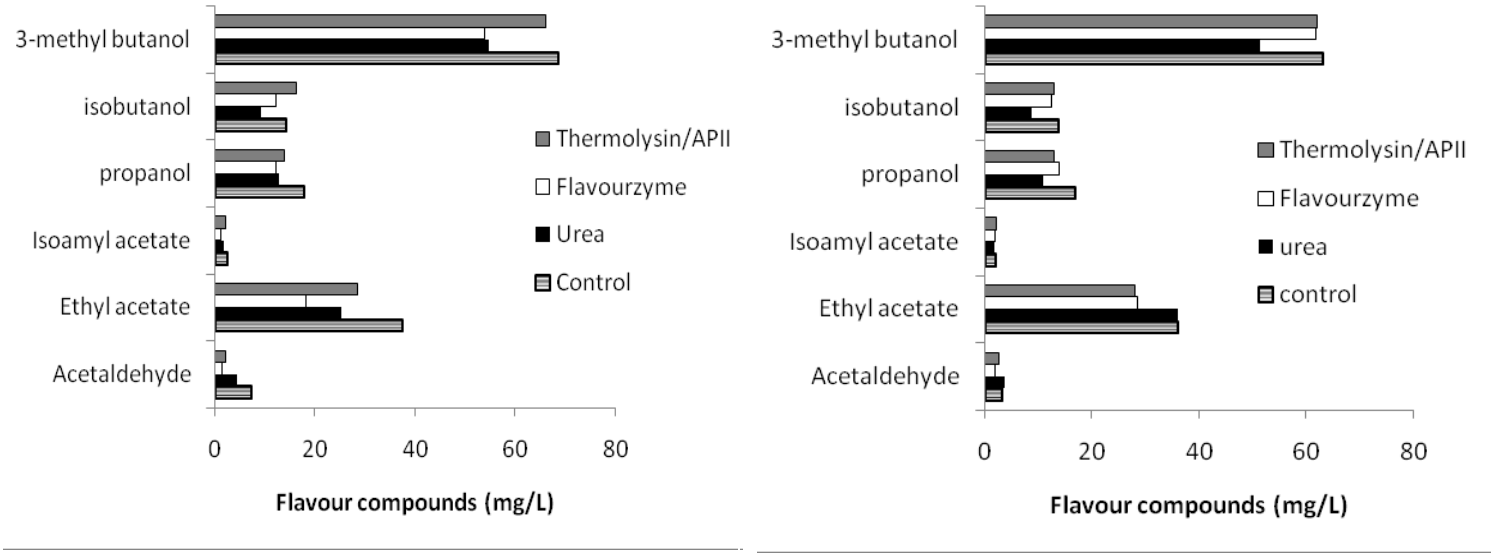


Figure 7-2. Concentration of the flavour and aroma components from the final beer. The values are reported after data normalization (correction) to the same ethanol content- 5 % (v/v). A. represents the flavour compounds from the glucose syrup supplemented fermentations and B. represents the flavour compounds from the maltose syrup supplemented fermentations.

The most pronounced difference between the flavour and aroma profile of the studied fermentations is significantly lower concentration of acetaldehyde in nitrogen supplemented fermentations compared to the control fermentations. This observation was especially pronounced for the fermentations with glucose syrup supplementation. While acetaldehyde concentration was 7.2 mg l^{-1} for the control, the acetaldehyde concentrations for the nitrogen supplemented fermentations were in the range of 1.5 to 4.2 mg l^{-1}. For the maltose syrup supplemented fermentations, the fermentations with enzyme supplementation had slightly lower acetaldehyde levels compared to the control. For the glucose syrup supplemented fermentations with nitrogen supplement, another interesting observation was the lower concentration of ethyl acetate. For the maltose supplemented fermentations, the fermentations with control and urea supplementation had similar concentrations of ethyl acetate, whereas lower concentrations of this compound were observed for the protease supplemented fermentations. While the isoamyl acetate concentrations were similar for all the fermentations with maltose syrup supplementations, for the glucose supplemented wort, isoamyl

acetate was present in slightly higher concentrations in the control fermentations compared to the nitrogen supplemented fermentations. Propanol was also present in higher concentrations for the control fermentations- 17.7 and 16.9 mg l^{-1} for the glucose and maltose supplemented fermentations, respectively, compared to the nitrogen supplemented fermentations, where propanol was in the range of 10.8 to 13.8 mg l^{-1}. There was no significant difference in the concentrations of isobutanol and 3-methyl-butanol among the studied conditions.

Discussion

The level of available nitrogen in beer fermentations is one of the most important factors assuring successful fermentation. Nitrogen supplementation in anaerobic beer fermentations is recommended as a way to achieve higher specific growth rate and to avoid sluggish and stuck fermentations (Beltran 2005, Batistote *et al.* 2006). Additionally, in brewing, the nitrogen metabolism is important as it is related to the flavour of the final beer. Nitrogen accumulation is affected by a number of factors such as pH, ethanol toxicity, temperature, carbon dioxide pressure, degree of aeration and plasma membrane composition (Henschke and Jiranek, 1994).

As wort is a highly complex medium and contains various nitrogen compounds, the regulation of amino acid metabolism during growth of brewer´s yeast on wort is highly complex. There is a global regulatory mechanism and in addition, there are intermediates and end products of the pathways leading to and from the individual amino acids that exert control by feed-back mechanism. As some amino acids are preferred by the brewer´s yeast more than others, the amino acids required for protein synthesis will not always come from the available exogenous amino acids sources. Despite the availability of certain amino acids in the media, biosynthesis of deficient amino acids will still be necessary. Lack of a particular amino acid may induce also many enzymes required for the synthesis of several other amino acids. This event involves increased transcription of more than 40 genes and it is referred to as general amino acid control (Hinnebusch, 1997). Typically, mainly the amino acids taken up early in the fermentation will support high specific growth rates, when supplied as sole nitrogen sources. However, in complex media such as wort and must, generally, the mixtures of amino acids support faster growth and fermentation rates than single compounds do.

It is known that the presence in the media of ammonia or glutamate causes a repression of the enzymes required for the catabolism of other amino acids. Some of the transporters, including general aminoacid permease (GAP) are repressed by ammonium ions, asparagine and glutamine present in the media (Grenson, 1992). GAP has maximum activity under the conditions of nitrogen limitation and it acts as a nitrogen scavenger. In *S. cerevisiae*, two classes of hexose carriers are recognised- high and low affinity carriers. In the presence of glucose or under nitrogen exhaustion, the maltose permease is irreversibly inactivated via the action of a protease (Busturia and Lagunas, 1986).

Based on the above facts, it can be hypothesized that for the studied fermentations with ammonium sulphate supplementation, ammonium induces repression of the uptake of other nitrogen sources, in particular amino acids which are essential for the yeast metabolism. Thus despite the availability of other nitrogen sources in the media, the brewer´s yeast can not utilise them and experiences conditions of nitrogen catabolite inactivation. In addition, the presence of glucose in the media also inhibits the maltose uptake. As a result, the maltose permease is irreversibly inactivated via the action of protease. Thus, for the ammomium sulphate supplemented fermentations in glucose syrup supplemented wort, nitrogen catabolite inactivation is the reason for the observed higher residual maltose and maltotriose concentrations and high residual FAN at the end of the fermentations.

In all cases of nitrogen supplementation, lower glycerol yields for the nitrogen supplemented fermentations with glucose syrup as an adjunct compared to the control were observed. It is known that synthesis of biomass results in intracellular net formation of NADH. Under anaerobic conditions, glycerol formation is the only possibility to oxidize NADH by regenerating NAD^+ and thus to maintain the redox balance (Van Dijken and Scheffers, 1986). Amino acid uptake is redox neutral, but the lesser demand for their biosynthesis in nitrogen supplemented fermentations will present a net savings in the demand for NAD^+ regeneration via glycerol formation.

Saccharomyces cerevisiae and brewer´s yeasts do not utilise proline, peptides exceeding five residues and proteins as a nitrogen source (Briggs et al., 2004). Thus, during the course of beer fermentations, even in conditions of nitrogen limitation, part of the nitrogen source remains unutilised from the wort.

The use of enzymes in the different steps of the brewing process is currently expanding with a main focus on improved wort attenuation, filtration and decrease of diacetyl formation from the final beer (Novozymes A/S 2008, Danisco A/S 2008). In the present study, the use of the multicomponent protease enzyme Flavourzyme allowed release of both exogenous and endogeneous nitrogen sources and enhanced the yeast growth and increased the specific ethanol productivity. Previous reports, where supplementation of wort with nitrogenous compounds in form of yeast extract, casamino acids, or single amino acids such as glutamic acid, have been investigated, resulted in enhanced yeast growth and increased specific ethanol productivity (Thomas and Ingledew, 1990).

The addition of proteases resulted in increase in the total FAN content, but did not result in significant increase in the amino acid concentrations. As beer batch fermentation is a dynamic process, amino acids are both synthesized and taken up and only the net result can be measured. As increased specific growth rate were found, the yeast would require an increased demand for nitrogen sources. One other possible explanation is that both the net amino acid release and the net amino acid consumption were higher. Alternatively, the observation that the total amino acid concentration levels remained similar is possibly also an indication that the used protease preferably releases oligopeptides instead of releasing individual amino acids.

Despite the fact that the combination of Thermolysin and Aminopeptidase II resulted in the highest amount of FAN, the fermentations where they were used did not show the most superior fermentation performance. Thermolysin supplementation experiments resulted in lower ethanol yield (**Table 7-3**) and longer fermentation time compared to the fermentations with other nitrogen sources, in addition to increased viscosity of the media and filtration problems together with increased flocculation (data not shown). Thermolysin supplementation for the maltose supplemented fermentations also resulted in the lowest specific growth rate observed in the screening experiment. As increased brewer´s yeast flocculation would result in decreased yeast surface for nutrient uptake, such fermentations will result in slower fermentation and longer fermentation times. The superior performance of the cultivations with combination of Thermolysin/Aminopeptidase II compared to single Thermolysin supplementation is possibly because the enzyme combination has both endo- and exopeptidase properties whereas Aminopeptidase II has only endopeptidase properties. Previous investigations of individually supplemented amino acids in wine fermentations have shown that addition of ammonium, glutamine, arginine, glutamate and asparagine increase the specific growth rate, while glycine,

threonine, isoleucine, valine and tryptophan are poor nitrogen sources resulting in low specific growth rate (Henschke and Jiranek, 1994). Most of the mentioned amino acids contributing to the lower specific growth rate contain side chains with hydrophobic character. Thermolysin is metalopeptidase, specifically catalyzing the hydrolysis of peptide bonds containing hydrophobic amino acids. Thus, there is possibility that this specific enzyme action might contribute to the increased viscosity of the fermenting wort. The increased viscosity of the media in turn contributes to decreased oxygen mass transfer and is also a possible reason for the lower observed specific growth rate.

The type and concentration of the supplemented nitrogen sources also greatly influence the flavour profile of the final beer. In the present study, the most significant differences for the studied flavour and aroma compounds for the nitrogen supplemented fermentations were lower concentrations of acetaldehyde and slightly lower concentrations of ethyl acetate compared to the control fermentations.

Acetaldehyde is an immediate precursor of ethanol, thus lower ethanol yield will result in lower acetaldehyde levels. Another possible explanation for the higher concentration of acetaldehyde in the control non supplemented fermentations is the activation of the general amino acid permease (GAP1). Gap 1 is a transport-protein and it is mainly active in the absence of easily utilizable nitrogen sources. Once the amino acid is in the cell, the amino group may be transferred by a transaminase to a carbon skeleton to form a new amino acid. The remaining carbon skeleton is then secreted or used to regenerate NAD^+. In this case the oxo-acid produced is decarboxylated to CO_2 and an aldehyde (Lewis & Young 2001; Briggs, 2004).

Ethyl acetate and isoamyl acetate are normally found in beer produced from average gravity- 10° - 12 °P in concentrations below their threshold values of 30 mg l^{-1} and 2 mg l^{-1} , respectively (Casey et al., 1985). One of the disadvantages of the high gravity brewing is associated with disproportionate higher levels of esters, particularly ethyl acetate and isoamyl acetate (Stewart, 2007). As amino acid uptake involves protein symport, it is possible that the intracellular pH of the yeast cells is affected. Thus, as a result of the pH change, the activity of esterases or acyltransferases could be modified, resulting in lower concentrations of ethyl acetate. This hypothesis is further supported by the fact that the fermentations with nitrogen supplementation resulted in slightly lower pH compared to the control fermentations (data not shown).

Conclusion

The present study showed that nitrogen supplementation for high gravity beer fermentation is beneficial for a successful fermentation performance. In addition, the choice of nitrogenous compounds as a supplement in beer fermentation in combination with the wort sugar composition is of great importance. Among the studied nitrogen sources, supplementation of a nitrogen source leading to best performance in terms of wort fermentability, specific growth rate, ethanol yield and flavor and aroma compound profiles was the protease supplementation with Flavourzyme.

Despite the fact that Flavourzyme supplemented fermentations resulted in ethanol yields similar to the control fermentations, its supplementation resulted in increased specific growth rate and thus increased specific ethanol productivity.

References

Analytica-EBC, European Brewery Convention, Section 8 Wort, Method 8.10. Verlag Hans Carl Getränke-Fachverlag: Nürnberg, Germany, 1998.

Batistote M, Helane da Cruz S, Ernandes, J. (2006) Altered Patterns of Maltose and Glucose Fermentation by brewing and Wine Yeasts Influenced by the complexity of nitrogen Source. J. Inst. Brew. 112: 84-91.

Beltran G (2005) Effect of low temperature and nitrogen content on wine yeast metabolism, PhD thesis, Univeritat Rovira i Virgili, Departament de Bioquímica I Biotecnologia, Facultat D'Enologia, Tarragona, Spain.

Boulton C, Quain D (2006a) High gravity brewing In: Brewing Yeast and Fermentation, 2nd edn. Blackwell Science Ltd., Oxford, UK, pp 60-63.

Briggs D, Boulton C, Brookes P, Stevens R (2004) Fermentation technologies. In: Brewing Science and Practice, Woodhead Publishing Limited, Cambridge, pp. 401-468.

Busturia A, Lagunas R (1986) Catabolite inactivation of the glucose transport system in Saccharomyces cerevisiae. J. Gen. Microbiol. 132: 379-385.

Casey G, Chen E, Ingledew W (1985) High gravity brewing: Production of high levels of ethanol without excessive concentrations of esters and fusel alcohols. Am. Soc. Brew. Chem. 43: 179-182.

Danisco A/S, web information. Available at http://www.danisco.com/cms/connect/corporate/products%20and%20services/product%20range/enzymes/brewing%20and%20distilling/brewing_distilling_enzymes_en.htm, accessed April 1, 2008.

De Rouck G., De Clippeleer J., Poiz S., De Cock J, van Waesberghe J, De Cooman L, Aerts G (2007) Proceedings of the 31st EBC Congress, Venice, 2007: 455-467.

Devantier, R (2005) Investigation of the mechanism behind the beneficial effect of protease addition to very high gravity ethanol fermentation of corn mash In: Saccharomyces cerevisiae in very high gravity ethanol fermentations using simultaneous saccharification and fermentation, PhD thesis, Technical University of Denmark, Kongens Lyngby, pp. 35-51.

Grenson M (1992) Amino acid transporters in yeast: structure, function and regulation. In: Molecular aspects of transport proteins. J. De Pont ed., Elsivier Science Publishers, London, pp.219-245.

Henschke P and Jiranek V, Metabolism of nitrogen compounds In: Wine Microbiology and Biotechnology, G. Fleet (ed), 1994, pp.77-165.

Herbert P, Santos L, Alves A (2001) Simultaneous quantification of primary, secondary amino acids, and biogenic amines in musts and wines using OPA/3-MPA/FMOC-Cl Fluorescent derivatives. J. Food Sci. 66:1319-1325.

Hinnebusch A (1997) In G.D. Fasman, Crit. Rev. Biochem., 3th edn, CRC Press, 21, pp. 277-317, Boca Raton, FL., USA.

Hjortshöj B, Avis J, Haukeli A, Kampers J, Olimans J, van den Berg R and Wacerbauer K (1992) Methylene blue staining, p. 6-7. EBC Analytica Microbiologica, vol. II, section 3. Fachverlag Hans Carl Postfach, Nuremberg, Germany.

Hofman-Bang J (1999) Nitrogen catabolite repression in *Saccharomyces cerevisiae*. Mol. Biotech. 12: 35-73.

Horak J (1986) Amino acid transport in eukaryotic microorganisms. Biochim. Biophys Acta, 864: 223-256.

Lewis M, Young T (2001) Fermentation biochemistry In: Brewing, 2nd edn. Aspen publishers Inc., New York, pp.319-338.

Lie, S (1973) The EBC-ninhydrin method for determination of free alpha amino nitrogen. J. Inst. Brew. 79:37-41.

Novozymes A/S, web information. Available at http://www.novozymes.com/en/MainStructure/ProductsAndSolutions/Brewing/Brewing.htm, accessed April 1, 2008.

O´Conor-Cox E, Paik J, Ingledew W (1991) Improved ethanol yields through supplementation with excess assimilable nitrogen. J. Ind. Microbiol. 8:45-52.

Stewart G (2007) The influence of high gravity wort on the stress characteristics of brewer´s yeast and related strains. Cerevisia 32:37-48.

Thomas, K, Ingledew, W (1990) Fuel alcohol production: Effects of free amino nitrogen on fermentation of very-high gravity wheat mashes. App. Environ. Microbiol. 56: 2046-2050.

Van Dijken J, Scheffers W (1986) Redox balances in the metabolism of the sugars by yeasts. FEMS Microbiol. Rev. 32: 199-225.

Walker, G. (1998) Yeast physiology and biotechnology, John Wiley & Sons Ltd., West Sussex, UK.

Wiame J, Marcelle G, Arst H (1985) Nitrogen catabolite repression in yeast and filamentous fungi In: Advances in Microbial Physiology, vol. 26, ed. Rose A, Tempest DW, Academic Press, London, Canada, pp. 2-88.

8

Revealing the beneficial effect of protease supplementation to high gravity beer fermentations using "–omics" techniques

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This chapter forms the basis for manuscript in preparation

Key words: High gravity, brewer´s yeast, transcriptome, metabolome, brewing enzymes

Abstract

The addition of sugar syrups to basic wort is a popular technique to achieve higher gravity in beer fermentations but results in diluted free amino nitrogen (FAN) content in the medium. The multicomponent protease enzyme Flavourzyme has beneficial effects on the brewer´s yeast fermentation performance during high gravity fermentations in terms of increased initial FAN value and higher FAN uptake, higher specific growth rate, higher ethanol yield and an improved flavour profile. In the present study, system biology tools- transcriptome and metabolome analysis were used to elucidate the effects on the addition of the multicomponent protease enzyme Flavourzyme and its influence on the metabolism of the brewer´s yeast strain Weihenstephan 34/70.

The present study underlines the importance of sufficient nitrogen availability during the course of beer fermentation. The applied metabolome and transcriptome analysis mapped the effect of the wort sugar composition on the nitrogen uptake. Both transcriptome and metabolome analysis revealed significantly higher impact on the studied protease addition for the maltose syrup supplemented fermentations while addition of glucose syrup to increase the gravity in the wort relieved the effect of glucose repression and further inhibited the effect of the protease addition.

Introduction

Despite the fact that beer brewing is an ancient trade, in today´s competitive market, there is constant demand for further process improvement. Some of the main targets for improvement in the brewing process are shorter fermentation times, higher wort fermentability and ethanol yield, reduced equipment and labor costs. High gravity brewing was introduced in the 1970s and has been gaining popularity in recent years.

High gravity brewing uses wort with a higher sugar concentration than normally used. Because of the high ethanol content produced, the end product is diluted to the required alcohol content. A popular approach for achieving higher sugar concentration is the addition of sugar syrups as adjuncts to wort with an average gravity (usually in the range of 12-14 °Plato). However, as sugar syrups do not contain any considerable amounts of nitrogen, their addition to the basic wort will dilute the free amino nitrogen (FAN) content present in the media. Thus to ensure presence of sufficient nitrogen and to avoid incomplete brewer´s yeast fermentation, it is a requirement that high gravity wort has higher free amino nitrogen content compared to lower gravity wort. Under anaerobic conditions, while FAN requirements for wort of 12 °Plato are in the range of 140-150 mg l^{-1}, wort with >18 °Plato requires FAN of 280 mg l^{-1} (O´Connor-Cox, 1991). To a certain extent, FAN requirements are also strain specific (Boulton and Quain, 2006).

Previously (Chapter 7), we characterized high gravity beer fermentations at 21 °P with increased available nitrogen content by the addition of various nitrogen sources - ammonium sulphate, urea or proteases.

The results from the physiological characterization showed that nitrogen supplementation generated by the addition of the multicomponent enzyme (Flavourzyme) with both endo and exo peptidase activities resulted in the best fermentation performance in terms of higher ethanol yield, specific growth rate and specific ethanol productivity in addition to high FAN utilization.

In today's brewing market the efforts are focused on maintaining low sale prices despite increases in raw material prices and on keeping constant prices for the end consumer while maintaining profits. Focused on these aspects, the enzyme producing companies offer new solutions by utilizing enzymes in the brewing industry. The main targets for the currently available enzymes in brewing

are focused on higher extract yields, improved attenuation control, longer beer filter cycle runs, shorter cooking cycles, and reduced beer losses and maturation time (Novozymes, 2008).

Proteases are commercially used for bioethanol production as a way to provide additional assimilable nitrogen for the yeast. Protease addition to the simultaneous saccharification and fermentation of whole milled cereals is known to improve final ethanol concentrations and volumetric productivities (Devantier, 2005). While addition of proteases is a commonly used practice for bioethanol production, studies of the addition of proteases as a way to enhance the free amino nitrogen in beer fermentation are very limited. In brewing, additions of proteases have further importance as the amino acid metabolism is related to the flavour and aroma profile of the final beer. The major classes of yeast derived flavor compounds are formed as by-products of the metabolism of sugars and amino acids (Briggs et al., 2004). Under fermentative conditions, some of the products from the amino acid metabolism might be released into the medium and contribute to the beer flavour.

To approach the problem with nitrogen limitations in the high gravity beer fermentations, in our previous study (Chapter 7), fermentations at 21°P achieved with the addition of either glucose or maltose syrup supplementation, both without and with addition of exogenous and enzymatically generated nitrogen sources has been performed. Among the studied nitrogen supplements, the multicomponent protease Flavourzyme showed the most superior fermentation performance in terms of increased initial FAN value and higher FAN uptake, higher specific growth rate, higher ethanol yield and improved flavour profile, in terms of lower acetaldehyde and ethyl acetate levels.

Transcriptomics allows studies of the mRNA expression levels on a genome wide basis and gene expression changes as a consequence of the changing environmental growth conditions can be studied. In general, transcriptome analyses provide first, insight about the active or inactive metabolic pathways under certain conditions and later, about the alternative types of regulation-transcription factor mediated versus post-translational regulation (Otero et al., 2007). The availability of commercial microarray platforms has established transcriptomics as a routinely used data set in studying many industrial organisms and it plays a central role in both metabolic engineering and other fermentation optimization strategies.

Metabolomics is another appealing “-omics” tool for quantitative characterization of the relationship between the genome and phenotype of the cells. It provides an important complement

to the mRNA and protein measurements in studying the cellular function. Metabolomics is an important tool in functional genomics and together with the structure of the metabolic network, the levels of the intracellular metabolites presents further information in understanding the flux regulation through the different metabolic pathways (Villas- Bôas et al., 2005a).

In this study, system biology tools such as transcriptome and metabolome analysis were chosen to further elucidate the effects on the addition of the multicomponent protease enzyme Flavourzyme and its influence on the metabolism of the popular brewer´s yeast strain Weihenstephan 34/70. In particular, the effects of Flavourzyme addition on the amino acids metabolism and flavour compounds formation was investigated.

Materials and methods

Strain

The flocculent bottom fermenting industrial lager beer yeast strain Weihenstephan 34/70 (Hefebank Weihenstephan, Freising, Germany) was used in this study. The strain was maintained as a frozen stock culture in 40 % (v/v) glycerol.

Fermentation setup

For the studied fermentations, wort with gravity corresponding to 21 °Plato was used. Higher gravity was achieved with either glucose or maltose syrup supplementation to the basic wort of 14 °Plato (Chapter 3). The fermentation setup was designed to simulate as closely as possible the larger scale beer fermentations.

Wort

All-malt wort with a starting gravity of 14.3 °P and pH=5.2, (Alectia, Denmark), was used. This wort contained 90 % carbohydrates of which the fermentable carbohydrates consisted of 4.4 % fructose, 12.5 % glucose, 66.5 % maltose and 16.7 % maltotriose (w/v). The wort also contained

non-fermentable carbon sources such as dextrins and β-glucan. $ZnSO_4.7H_2O$, was added to a concentration of 0.1 ppm Zn. In order to adjust the wort to higher gravities - 21 °Plato, highly fermentable syrups - Clearsweet® 95% Refined Liquid Dextrose Corn Syrup (95.5 % glucose, 2.5 % maltose, 1 % maltotriose, 1 % higher saccharides, present in % dry basis (w/w) and Satin Sweet® 65 % High Maltose Corn Syrup (70 % maltose, 18 % maltotriose, 9 % higher saccharides, 2 % glucose, present in % dry basis) were used as adjuncts. Both syrups were kindly provided from Cargill Nordic A/S. The resulting sugar composition of the different fermentation media used in this study is summarized in Chapter 3. Prior to inoculation, the wort was oxygenated with air until it reached 100 % saturation.

Fermentation conditions

For the pre-cultures, the yeast from the stock culture was propagated on YPD plates at 30 °C for four days. A single yeast colony was transferred to 20 ml of 14 °P wort in a sterile 50 ml Falcon tube and incubated at 25 °C in a rotary shaker at 150 rpm. After 48 hours, the preculture was transferred to a 500 ml shake flask with 375 ml of fresh wort and incubated for 72 hours.

All fermentations were performed in 2.2 liter bioreactor (Biostat B5; Braun Biotech International, Melsungen, Germany) with a working volume of 1.5 liter. Dissolved oxygen was monitored with an autoclavable polarographic oxygen electrode. The fermentors were connected to Braun Biotech Multi-Fermenter Control System (MFCS) for data acquisition. Silicone based antifoam agent FD20P in a concentration of 0.1 ml/L (Basildon Chemicals, England) with food grade quality was used in the fermentations. The reactors were inoculated with a volume of pre-culture, corresponding to 1×10^7 cells/ml. During the cultivation the temperature was maintained at 14 °C and the stirring was set to 90 rpm. Prior to sampling the stirring was increased to 300 rpm for 2 min. The higher stirring allowed better mixing and homogenization of the media and ensured representative sampling. The pH was recorded on-line, but not controlled. After the fermentation was completed, the whole fermentation broth was transferred to a sterile vessel and stored for 14 days at 0 °C, for further maturation. Detailed physiological characteristics of the performed cultivations have been described previously (Chapter 3).

Calculations of growth characteristic parameters

The specific growth rate was determined as the slope from the linear function of the ln (natural logarithmic function) of the cell number (cells/ml) and the fermentation time (h) during the exponential growth phase. The yield coefficients were determined as the slope from the linear regression on the corresponding pairs of substrate (total saccharides) and product concentration (glycerol and ethanol).

Protease supplementation

In order to increase the available free amino nitrogen content in the studied high gravity beer fermentations, the enzyme Flavourzyme was used as a supplement in a concentration of 60 ppm. Flavourzyme is a commercially available enzyme produced by Novozymes A/S. Previously, Flavourzyme has been commercially used to boost the nitrogen supplementation in bioethanol fermentations. It is a fungal multicomponent enzyme produced from *Aspergillus oryzae* with both endo- and exo- peptidase activities. The optimal temperature and pH for this enzyme complex is 50 ºC and pH 5-7, respectively.

Enzyme hydrolysis

In order to determine the activity of the enzyme Flavourzyme and to investigate whether an increase in the gravity or change in the wort sugar composition influences its activity, the hydrolysis efficiency of Flavourzyme in wort at three different gravities was examined. The three different wort compositions were: 14°Plato wort, 21°Plato wort adjusted with glucose syrup and 21°Plato wort adjusted with maltose syrup. The hydrolysis was performed in 1 L autoclaved shake flasks with working volume of 800 ml of the selected wort. The enzyme Flavourzyme was added to each of the flask in a concentration of 60 ppm. As controls, shake flask hydrolysis with no enzyme addition were used. The shake flasks were placed on a shaking table with an agitation of 90 rpm and a temperature of 14°C, conditions that resemble those prevailing during the brewing experiment. For the sampling, 10 ml samples from each of shake flasks were taken daily. Each hydrolysis lasted for two weeks and was done in duplicates.

Sampling

In order to ensure homogenous composition of the cultivation liquid, prior to each sampling, stirring was increased to 300 rpm. Both transcriptome and metabolome samples were stored at -80 °C until further treatment. Samples for analysis of sugars and alcohols were collected on a regular basis every 24 hours throughout the fermentation. For measuring the free amino nitrogen content from the fermentations, samples were collected from the first and the final day of the primary fermentation. For all of the above analyses, 2-10 ml of fermentation samples were withdrawn from the fermentor, immediately filtered through a Cameo 0.20 µm pore size acetate/glass filters (Sartorius AG, Germany) and stored at -20 °C prior to analysis.

For the cells count determination, samples were also collected daily. For the transcriptome analyses 20 ml samples were taken both from the early exponential and from the stationary phase. For the metabolome analyses, 10 ml samples were taken in the early phase and 20 ml samples, respectively were taken in the stationary phase.

Free amino nitrogen (FAN)

The levels of free amino nitrogen (FAN) of the unfermented worts and from the last day of the beer fermentation were determined using the ninhydrin method at 570 nm (Lie, 1973; EBC method). Glycine was used as a standard.

HPLC analysis

A Dionex Summit HPLC system (Synnyvale, CA) was used for analysis of sugars and metabolites from the extracellular medium. All metabolites were detected refractometrically (Waters 410 Differential Refractometer Detector, Millipore Corp., Milford, MA) after separation on an Aminex HPX-87H column (Biorad, Hercules, CA) at a temperature of 60 ^{o}C using 5 mM H_2SO_4 as eluent. To allow the separation of the sugars with different degrees of polymerization, two Aminex columns were mounted in series with isocratic elution at 0.40 ml/min. External standards of maltotriose (DP3), maltose (DP2), glucose (DP1), fructose (DP1), glycerol and ethanol were used.

Metabolome analysis

After withdrawal from the fermentor, the samples were rapidly quenched into 20 ml precooled (-40 °C) 72 % methanol. Cells were centrifuged at 10 000 x g for 20 minutes in -20 °C to separate them from the quenching solution. Further on, the intracellular metabolites were extracted using chloroform: methanol: 3 mM Pipes buffer (pH=7) extraction (Villas-Boas et al., 2005). Following extraction, the samples were lyophilized using a Christ-Alpha 1-4 freeze dryer.

Following lyophilisation, the samples were resuspended in 200 µl of 1% (w/v) NaOH solution and derivatised using the methodology by Villas-Bôas et al., 2003. In order to decrease the matrix effect in the extracellular samples containing a high concentration of maltodextrins (sugars), the samples were resuspended in 2 ml of NAOH solution. As external standards, amino acid standards (Sigma) at two different levels were used. As internal standards, 20 mM of EDTA and 30 mM of chlorophenylalanine were used. Samples were normalised by the amount of intracellular standards and by the cell number and expressed as normalized peak area. Intra- and extracellular metabolites belonging to the group of amino acids and non-amino organic acids were analysed by GC-MS. GC-MS analysis was performed with a Hewlett-Packard system HP 6890 gas chromatograph coupled to a HP 5973 quadrupole mass selective detector (EI) operated at 70 eV. For the analyses, column J&W1701 column with size (30m ×250 µm × 0.15 µm) was used (Folsom, CA). The used GC-MS program has previously been described (Panagiotou et al., 2007). Peak detection was conducted with AMDIS (NIST, Gaithersburg, MD) using default parameters.

Transcriptome analysis

Probe preparation and hybridization to arrays

Samples for RNA isolation were taken in duplicates during the stationary phase of the fermentations. For each sample, 20 mL of culture were sampled into 50 mL tubes containing 20 mL crushed ice and immediately centrifuged at 4000 rpm for 5 min at 4 °C. The supernatant was discarded and the pellet was frozen instantaneously in liquid nitrogen and stored at -80 °C. Total RNA was extracted using RNeasy Mini Kit (Qiagen), according to the protocol for total RNA

isolation from yeast. The quality and the integrity of the extracted total RNA was analyzed using an Agilent Bioanalyzer 2100 (Agilent technologies Inc., USA) and RNA 6000 Nano LabChip kit. The cDNA and cRNA syntheses, labeling and cRNA hybridization on the oligonucleotide Yeast Genome 2.0 Array (Affymetrix, CA) were performed as described in the Affymetrix GeneChip® expression analysis manual (Affymetrix, 2000). GeneChip® Hybridization Oven, Fluidics Station FS-450 and 3000 7G Scanner were used for array hybridization, washing, staining and scanning.

Data acquisition and gene expression analysis

Affymetrix Microarray Suite v5.0 was used to generate CEL files from the scanned microarrays. Data analysis was performed by using the statistical open source language R (Gentleman et al., 2005). Data preprocessing was carried out by using the Robust Multichip Average (RMA) method (Irizarry, 2003). The function *rma* is contained in the R/affy package (Gautier, 2004) and it implements RMA by correcting the Perfect Match (PM) probes, performing quantile normalization (Bolstad et al., 2003) and calculating the expression measure by using median polish.

In order to select genes whose expression levels were related to the experimental factors, Analysis Of Variance (ANOVA) was performed. The 2x2 experimental design comprises two main factors ('wort type' and 'protease supplementation'), each having two levels ('glucose wort/maltose wort' and 'protease supplemented/protease not-supplemented'). The ANOVA model was fitted in order to identify significantly changed gene expression levels with respect to the two above mentioned factors, as well as, the interaction term ('wort type*protease supplementation'). The *p*-values were corrected for multiple testing by applying the False Discovery Rate (FDR) methodology (Benjamini and Hochberg, 1995) and genes were selected by imposing a cut-off value of 0.05. Furthermore, as regards to the gene lists associated with the two main factors, only genes with $|\log_2(\text{fold change})| > 1.301$ were considered. Genes selected within the interaction term were further investigated in order to find out which genes showed differences in each of the four possible factor level combinations: 'glucose wort*protease supplemented', 'glucose wort*protease non-supplemented', 'maltose wort*protease supplemented' and 'maltose wort*protease non-supplemented'. The identification of these genes was achieved by using the template match method and the R/code (Pavlidis, 2003). Moreover, gene ontology (GO) process terms of the selected genes were determined using GO Slim Mapper tools (Saccharomyces Genome Database (SGD)) with significance at $P < 0.01$.

Reporter Regulators (transcription factors) analysis

Reporter regulators, also named reporter transcription factors or TFs, were determined using the software and regulatory network of Oliveira et al., 2008. The software is based on reconstructed graphs covering each known transcription factor or regulatory protein, connected to all genes known to be effected by these proteins from the Yeast Protein Database (YPD). Using the entire gene expression set, reporter regulator analysis was used to identify the transcription factors and their regulatory pathways that were most significantly affected by the addition of protease in the 21 °P glucose or maltose syrup supplemented fermentations.

In brief, for the Reporter TFs algorithm, gene expression data from the significance of change for t-test comparison was used as an input together with the topology of the bio-molecular interaction networks (physical or functional interactions) represented as a graph, where each gene of interest is associated with a transcription factor (TFs). The basic regulatory principle behind it is that a perturbation or a response to perturbation might trigger regulatory response beginning from the first neighbors of the affected nodes. The algorithm identifies groups of neighbor genes (genes associated with certain feature), that are significantly and collectively co-regulated compared to the background. This concept is extended to any n^{th} degree neighbors. The algorithm does not require accounting for the significant changes at the level of each node (for example, the transcript of a gene). Gene expression, in the form of Z-score is then mapped onto the "gene nodes" of the graph. The score of each feature can be calculated based on the score of its neighbor's "gene nodes" and reporter features are then those features with a Z-score above the selected cut-off (Oliveira et al., 2008).

Results

During the course of high gravity beer fermentations, brewer's yeast is exposed to a number of stressful conditions such as high osmotic pressure caused by the high glucose concentrations in the beginning of the fermentations and ethanol stress, imposed by the elevated ethanol concentration levels towards the end of the fermentations. Additionally, an increase in the gravity by the addition of sugar syrups to the media results in lower free amino nitrogen concentrations than the minimum required, thus brewer´s yeast is exposed to an additional stress, caused by nitrogen limitation and

limitations in the level of other nutrients, resulting in restricted growth (Gibson et al., 2007). In order to further investigate the effect of protease addition and its influence on the brewer´s yeast metabolism, detailed intra and extracellular metabolome and transcriptome analyses were performed in samples collected from the early exponential and from the stationary phase of the fermentations.

Enzyme hydrolysis

In order to determine the activity of the enzyme at the conditions prevailing during beer fermentation, enzyme hydrolysis experiments were performed.

With an optimum temperature at 50 ºC and a pH of 5-7, the lager beer fermentations at 14 ºC, with a pH ranging from around 5.2 to 4 during the course of the fermentations, provided harsh conditions for the enzyme to work. According to the Flavourzyme product sheet, at the conditions imposed during lager beer fermentation, the enzyme will be functioning with approximately 40 % of its maximum activity. To determine the activity of Flavourzyme during the course of lager beer fermentations, Flavourzyme hydrolysis was performed at conditions resembling the conditions of lager beer fermentations. The results of the FAN analysis of the first and the last sample of the enzyme hydrolysis experiments are presented in **Table 8-1**.

Table 8-1. Results from the FAN measurements of initial and final samples collected during enzyme hydrolysis assays.

Plato			**Average FAN (mg l^{-1})**	**Standard deviation (%)**
14 °P	**Control**	**Initial**	239	0.8
		Final	249	0.4
	Flavourzyme supplementation	**Initial**	229	0.1
		Final	246	4
21 °P Gl	**Control**	**Initial**	200	3
		Final	208	1.4
	Flavourzyme supplementation	**Initial**	218	1.8
		Final	242	2.5
21 °P M	**Control**	**Initial**	210	1.0
		Final	212	2.4
	Flavourzyme supplementation	**Initial**	212	3.8
		Final	251	4.8

[a] *The values are average of duplicate measurements with std < 10%*

The hydrolysis experiments showed significantly higher increase in the FAN content, for the Flavourzyme supplementation (25 to 40 mg/L), while variations at the FAN value for the control fermentations remain within the measurement standard deviation of 10%.

Physiological characterisation of the Flavourzyme supplemented fermentations

Previous characterisation of the Flavourzyme supplemented fermentations has shown that higher specific growth rates, but similar overall ethanol yield were observed compared to the non supplemented fermentations resulted (Chapter 7).

Both for the glucose and maltose supplemented fermentations with Flavourzyme addition, the specific growth rate was 0.074 (h^{-1}), compared to 0.050 (h^{-1}) and 0.064 (h^{-1}) for the glucose and maltose syrup supplemented fermentations, respectively, without enzyme supplementations. For the glucose syrup supplemented fermentations, the ethanol yield was 0.48 g/g both for the non-supplemented and Flavourzyme supplemented fermentations, while the glycerol yield was 0.026 and 0.024 (g/g), respectively. For the maltose supplemented fermentations, ethanol yield was 0.49 (g/g) for the control and 0.47 (g/g) for the Flavourzyme supplemented fermentations, while the glycerol yield remained the same in both cases- 0.018 g/g. Despite the similar or slightly lower ethanol yield for the Flavourzyme supplemented fermentations, due to the higher specific growth rate, the specific ethanol productivity (calculated from the yield coefficients multiplied by the growth rate) of those fermentations was higher. The Flavourzyme supplemented fermentations also showed improved wort fermentability. While the non supplemented fermentations resulted in final gravities of 5.17 and 4.29, for the glucose and maltose syrup supplementation, respectively. The same fermentations with Flavourzyme addition had final gravities of 4.99 and 3.47, respectively, pointing to an improved utilization of the nitrogen available in the wort. Furthermore, supplementation with Flavourzyme also resulted in a shorter lag phase prior to ethanol production (**Figure 8-1**).

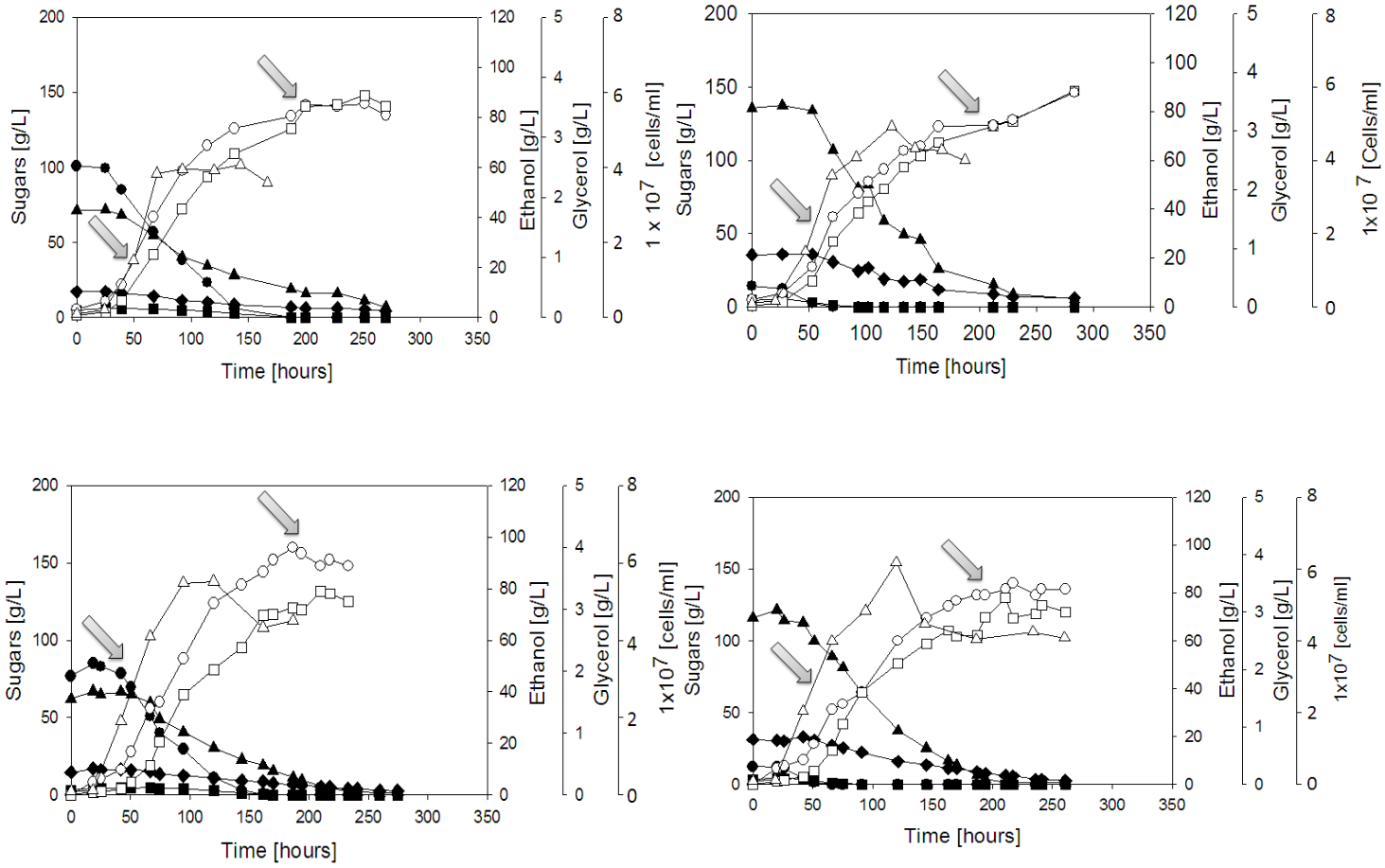

Figure 8-1. A. Control fermentations with glucose syrup supplementation; B. Control fermentations with maltose syrup supplementation; C. Glucose syrup supplemented fermentations with Flavourzyme additions; D. Maltose syrup supplemented fermentations with Flavourzyme addition. The arrows in the figures correspond to the sampling points for metabolome and transcriptome analysis of the studied fermentations. The symbols in the graphs represent the concentrations of: "—●—" glucose, " —■— "fructose, "—▲—" maltose, " —◆— " maltotriose, " —□— " ethanol,"—○— " glycerol and " —△— " cells/ml.

In order to further investigate the effects of the multicomponent enzyme Flavourzyme on the metabolism of the lager brewer´s yeast strain Weihenstephan 34/70, following the physiological characterization, samples for transcriptome analysis and intra- and extracellular metabolome analysis from the early exponential phase (around 50 to 70 fermentation hours) and from the stationary phase (around 200 to 220 fermentation hours) were collected (indicated by arrows in **Figure 8-1**.

Metabolome analysis

Metabolites are the end product of cellular regulatory processes and they play a very important role in connecting many different pathways that operate within a living cell. Metabolome analysis is complicated since it includes all small molecules in a biological system. In addition, metabolites are heterogeneous, have different types of structures, functional groups, physicochemical properties and concentrations and many of them are still unknown (Villas-Bôas et al, 2004). Thus, the levels of metabolites can be regarded as the ultimate response of an organism to genetic alterations or environmental influences.

In this study, the principle of alkylation reaction using methyl chloroformate (Villas-Bôas, 2005) was used to enable simultaneous separation, detection and quantification of both intracellular and extracellular metabolites, belonging to the groups of amino acids and non-amino organic acids as well as their derivatives. From the approximately 600 metabolites included in the genome-wide metabolic reconstruction for yeast (Förster et al., 2003), 40% are amines, amino acids and organic acids. Out of those, the applied method used an in-house developed MS library consisting of 75 metabolites playing a major role in the central carbon metabolism and amino acid biosynthesis.

Intracellular

In total, 39 intracellular metabolites were determined from the early exponential and from the stationary phase of the studied fermentations.

Table 8-2. Fold change of the intracellular amino acids and amino acid intermediates compounds of the maltose syrup versus glucose syrup supplemented fermentations.

	Maltose/ Glucose Exponential phase		**Maltose/ Glucose Stationary phase**	
	Control	**Flavourzyme supplementation**	**Control**	**Flavourzyme supplementation**
Alanine	**0.19**	**1.18**	**4.63**	**1.22**
Glycine	**0.05**	**0.22**	**5.46**	**1.40**
2-aminobutyric acid	**0.13**	**0.49**	**0.29**	**1.61**
Valine	**0.12**	**1.15**	**6.06**	**1.49**
Leucine	**0.14**	**1.97**	**3.61**	**1.35**
Isoleucine	**0.38**	**2.74**	**4.01**	**0.95**
4-amino-n-butyric acid	**-**	**1.20**	**1.41**	**0.85**
Proline	**0.13**	**0.82**	**0.55**	**1.11**
Threonine	**0.42**	**1.00**	**0.89**	**0.39**
Serine	**0.26**	**1.75**	**1.44**	**0.73**
Asparagine	**1.02**	**1.37**	**1.82**	**1.00**
Methionine	**0.74**	**1.78**	**-**	**-**
N-acetyl-L-glutamate	**-**	**-**	**0.37**	**0.54**
Phenylalanine	**0.29**	**1.40**	**10.7**	**1.14**
Trans-4-hydroxyproline	**-**	**-**	**-**	**1.87**
Ornithine	**-**	**0.35**	**1.02**	**0.90**
Lysine	**0.35**	**0.65**	**8.95**	**1.99**
Glutamine	**-**	**-**	**-**	**2.50**
Histidine	**0.29**	**1.31**	**13.2**	**1.03**
Tyrosine	**0.50**	**1.01**	**3.50**	**0.84**
Triptophan	**0.42**	**1.14**	**7.38**	**1.19**
Cystathionine	**0.27**	**0.46**	**13.8**	**1.29**
aspartic acid	**0.27**	**1.10**	**3.26**	**1.69**
glutamic acid	**0.19**	**1.76**	**3.72**	**1.00**

The cells in gray represents an increase in the fold change between the Flavourzyme supplemented versus non-supplemented fermentations for the respective fermentation phase. The symbol "-"indicates that the metabolites were not present in the studied conditions.

Comparison between the glucose and maltose syrup supplemented fermentations with protease supplementation showed higher concentrations for most of the amino acids and organic acids for the maltose syrup supplemented fermentations with protease addition. The effect of the addition of Flavourzyme was especially pronounced for the early exponential phase of the studied fermentations. From the early exponential phase of the control fermentations only asparagine was present in higher level for maltose syrup supplemented fermentations. For the samples from the exponential phase, Flavourzyme addition in the maltose syrup supplemented fermentation showed an increase in the fold chance of fifteen of the studied amino acids (**Table 8-2**). For the early exponential phase, the fold change increase for most of the amino acids was in the range from 1 to 2, with the exception of isoleucine, with fold change increase of 2.74. For the stationary phase of the control fermentations, in total fifteen amino acids were present in higher fold change for the maltose syrup supplemented fermentations with a fold change in the range from 1 to 13.8. For the stationary phase of the control fermentations, the highest fold change for the maltose syrup supplemented fermentations compared to the glucose syrup supplemented ones was observed for the intermediate of cysteine- cystathionine(13.8), histidine (13.2), phenylalanine(10.7) and lysine (8.95) (**Table 8-2**).

Table 8-3. Fold change of the intracellular organic acids of the studied fermentations of the maltose syrup versus glucose syrup supplemented fermentations. The cells in grey represent the increase in the fold change for the studied metabolites between the Flavourzyme supplemented versus non-supplemented fermentations for the respective fermentation phase.

	Maltose/ Glucose Exponential phase		**Maltose/ Glucose Stationary phase**	
	Control	**Flavourzyme supplementation**	**Control**	**Flavourzyme supplementation**
Pyruvic acid	**-**	**3.63**	**-**	**0.68**
Malonic acid	**-**	**0.13**	**-**	**0.35**
(3S)-3-Methyl-2-oxopentanoic acid	**-**	**0.36**	**-**	**0.61**
fumaric acid	**-**	**1.16**	**-**	**0.96**
malic acid	**-**	**1.16**	**-**	**0.96**
succinic acid	**0.10**	**1.57**	**1.36**	**0.62**
citramalic acid	**0.00**	**1.03**	**1.69**	**0.45**
nicotinic acid	**0.29**	**3.09**	**0.34**	**1.10**
2-isopropylmalic acid	**-**	**-**	**0.39**	**1.30**
2-oxoglutaric acid	**-**	**0.84**	**0.49**	**2.70**
citric acid	**0.20**	**1.74**	**1.46**	**1.59**
cis-Aconitic Acid	**0.35**	**1.98**	**3.75**	**1.27**
pyroglutamic acid	**0.26**	**-**	**-**	**-**
isocitric acid	**0.09**	**0.64**	**0.10**	**1.23**
cumaric acid	**-**	**0.71**	**-**	**0.56**
5-hydroxymethyl-2-furaldehyde	**-**	**0.78**	**-**	**0.46**
glutaric acid	**-**	**0.01**	**-**	**0.71**
glyceric acid	**-**	**1.71**	**-**	**0.91**

All of the studied organic acids from the early exponential phase of the control fermentations were present in higher concentrations for the glucose syrup supplemented fermentations compared to the maltose syrup supplemented ones (**Table 8-3**). Addition of Flavourzyme to the maltose syrup supplemented fermentations resulted in increase in concentrations for most of the TCA cycle intermediates. While for most of the organic acids the increase in the fold change was in the range of 1 to 2, pyruvic acid and nicotinic acid showed highest fold change increase- 3.63 and 3.09, respectively. In the stationary phase, the control fermentations with maltose syrup supplementation resulted in higher fold increase in the range of 1.36 to 3.72 for succinic, citramalic, citric and cis-aconitic acids, respectively. Addition of Flavourzyme to the maltose supplemented fermentations resulted in higher fold change for nicotinic, isocitric, cis-aconitic, 2-isopropylmalic, citric and 2-oxoglutaric acids. Their fold change increase was in the range of 1.10 to 2.70.

Extracellular metabolites

In total, 14 extracellular metabolites were determined from the early exponential and from the stationary phase of the studied fermentations. In general, fewer compounds were found extracellularly than intracellularly.

Table 8-4. Fold change of the extracellular amino and organic acids detected from the stationary phase of the studied fermentations. The symbol "-"indicates that the compound was not present in at least one of the studied conditions. The cells in grey represent the increase in the fold change for the respective metabolites between the Flavourzyme supplemented versus non-supplemented fermentations.

	Maltose/ Glucose		**Glucose/ Maltose**	
	Control	**Flavourzyme supplementation**	**Control**	**Flavourzyme supplementation**
Alanine	**>20**	**1.97**	**-**	**0.51**
Valine	**>20**	**2.09**	**-**	**0.48**
Leucine	**>20**	**9.36**	**-**	**0.11**
Isoleucine	**>20**	**9.36**	**-**	**0.11**
Proline	**2.51**	**0.29**	**0.40**	**3.43**
Citric acid	**0.95**	**0.52**	**1.05**	**1.94**
Phenylalanine	**>20**	**1.48**	**-**	**0.67**
Tyrosine	**>20**	**>20**	**-**	**-**
succinic acid	**0.45**	**0.23**	**2.24**	**4.28**
Cis-Aconitic Acid	**-**	**-**	**>20**	**6.62**
2-oxoglutaric acid	**-**	**-**	**>20**	**-**
Pyruvic acid	**>20**	**0.36**	**-**	**2.79**
Citramalic acid	**-**	**-**	**-**	**>20**
2-isopropylmalic acid	**>20**	**-**	**-**	**-**

Comparison of the determined extracellular metabolites from the stationary phase between the maltose and glucose syrup supplemented fermentations without protease supplementation, showed significantly higher concentrations of amino acids and organic acids involved in the pyruvate metabolism- alanine, valine, leucine, pyruvic acid and 2-isopropylmalic acid. In addition, phenylalanine and tyrosine involved in the phosphoenolpyruvate metabolism and isoleucine were also present in significantly higher concentrations for the maltose syrup supplemented fermentations (**Table 8-4**). For the glucose supplemented fermentations without protease supplementation, the TCA cycle intermediates- citric acid, succinic acid, cis-aconitic acid and 2-oxoglutaric acids were present in significantly higher concentrations.

Addition of Flavourzyme to the maltose syrup supplemented fermentations resulted in further increases of the alanine, valine, leucine, isoleucine and phenylalanine concentrations (**Table 8-4**). Addition of Flavourzyme to the glucose syrup supplemented fermentations resulted in further increases of the concentrations of the TCA intermediates- citric, succinic, pyruvic acid as well as increase in the concentration of citramalic acid.

Transcriptome analysis

To study the effect on the addition of the multicomponent protease Flavourzymes on the metabolism of the brewer´s yeast Weihenstephan 34/70, genome wide transcription profiles from the stationary phase of the studied fermentations were collected. To quantitatively determine the genes with significantly changed expression, ANOVA analysis was performed.

Based only on the type of sugar syrup used (glucose rich versus maltose rich), at a p-value of 0.05, ANOVA test revealed in total 311 significantly changed genes. Of those, 204 were downregulated and 107 were upregulated in the glucose syrup supplemented fermentations. The levels of the fold change for the significantly changed genes were in the range from -4 to 3.4. Among the down regulated genes, the most significantly enriched GO terms revealed overrepresentation for the following categories: organelle organization, RNA metabolic process, translation, transcription, transport, cell cycle as well as response to stress, carbohydrate and lipid metabolism. Of the down regulated genes, 6 genes, representing 2.9% of cluster frequency were involved in the amino acids related metabolism and protein catabolism (**Table 8-1S**, supplementary information). The six genes were involved in amino acids biosynthesis (ECM17), glycine catabolism (GRS1), branch chain

amino acids- leucine, isoleucine, valine synthesis and catabolism (ILV6, ARO80), tyrosine (MSY1) and phenylalanine (MSF1) synthesis. For the up-regulated genes, the GO overrepresented terms included transport, response to stress, RNA metabolic process, organelle organization and transcription as well as amino acid and amino acid derivative metabolic processes, protein catabolic processes, carbohydrate and lipid metabolism. In total, 9 genes, representing 8.4% cluster frequencies were involved in the amino acid and protein catabolic processes (Table 1, supplementary information). Those genes were mainly involved in the glutamate (GDH3, CIT2), histidine (HIS1), methionine (ADI1, STR3) and asparagine (ASN1) biosynthesis as well as glycine (GCV1) and proline (PUT1) catabolism.

Genes with unknown biological function were 16 of the up-regulated and 34 of the down-regulated genes. Possible reason for observing genes with unknown biological function is either that they are genes coming from the non-*S. cerevisiae* part of the brewer´s yeast genome or because of the weak expression under the studied conditions; lack of annotation for those genes appeared.

Based on the effects of the enzyme addition itself, at a p-value of 0.05, ANOVA tests of the four studied conditions revealed 183 significantly changed genes. Of those, 87 genes were downregulated and 82 were upregulated in the presence of Flavourzyme. The GO overrepresented terms for the downregulated genes included translation, organelle organization, RNA metabolic processes, ribosome biogenesis, transport, transcription and cell cycle (Table 2, supplementary material). Six of those genes were involved directly into the amino acid metabolism. They encode the biosynthesis of leucine (LEU2), histidine (HIS1, HTS1), lysine (LYS12), isoleucine (ILV3) and phenylalanine-t-RNA synthetase (MSF1), respectively.

For the upregulated genes, the GO terms included transport, organelle organization, response to stress, RNA metabolic process, protein modification process, transcription, DNA metabolic process.

Among them, the genes involved in the amino acid metabolism were cystathionine beta-lyase (STR3), converting cystathionine into homocysteine, proline oxidase (PUT1) and acireductone dioxygenease (ADI1), involved in the methionine salvage pathway (**Table 8-2S**, supplementary material).

At a p-value of 0.05, the interaction effect, accounting for both the addition of Flavourzyme as a nitrogen supplement to the control fermentations and the type of sugar syrup used to increase the

gravity, resulted in a significant change of the transcriptional response for only 6 genes for the glucose supplemented fermentations. While for the maltose supplemented fermentations, in total 86 genes were significantly changed. Two of the significantly changed genes for the glucose syrup supplemented fermentations were putative proteins with unknown function, YJR011C and YCR016W, respectively. The four genes with known function- RME1, MCD4, SLD2 and SWD2 were involved in cell growth related processes such as transcription, cell cycle, meiosis, RNA and DNA metabolic processes (**Table 8-3**). Among the four genes, only MCD4 was upregulated while the rest of the genes were downregulated.

Among the 86 significantly changed genes for the maltose supplemented fermentations with Flavourzyme addition, 41 genes were downregulated and 45 genes were upregulated. In order to determine significantly enriched Gene Ontology (GO) process terms within the significantly changed genes based on the interaction effect of the type of sugar syrup and addition of protease, Saccharomyces Genome Database (SGD) - GO tools with significance at $p< 0.05$ were used.

The most overrepresented GO terms among the upregulated genes with known function were transport and cell growth related processes such as RNA metabolic process, transcription, DNA metabolic process (**Table 8-5**). 11 of the 45 upregulated genes were ORF with unknown function. Three of the upregulated genes - ARG82, ARO80 and MET13, were directly involved in amino acid metabolism. ARG82 is inositol polyphosphate multikinase (IPMK), regulating arginine-, phosphate-, and nitrogen-responsive genes; ARO80 is activating transcription of aromatic amino acid catabolic genes in the presence of aromatic amino acids and MET13 is a major isozyme of methylenetetrahydrofolate reductase, involved in the methionine biosynthesis pathway. Among the downregulated genes, the most overrepresented GO terms were also cell growth related processes such as RNA metabolic process, organelle organization, transcription, ribosome biogenesis and translation. Three of the downregulated genes- FRS2, VAS1, HTS1 were also involved in cellular amino acid and derivative metabolic process. FRS2 is alpha subunit of cytoplasmic phenylalanyl-tRNA synthetase, VAS1 is mitochondrial and cytoplasmic valyl-tRNA synthetase and HTS1 is cytoplasmic and mitochondrial histidine tRNA synthetase.

Table 8-5. GO annotation based on the biological process ontology for the significantly changed genes in maltose syrup supplemented fermentations with Flavourzyme addition.

GOID	GO term	Gene hits	Cluster Frequency
Up-regulated (45)			
	biological process unknown	11	24.4%
	transport	8	17.8%
	RNA metabolic process	7	15.6%
	transcription	7	15.6%
	response to stress	7	15.6%
	DNA metabolic process	7	15.6%
	protein modification process	6	13.3%
	vesicle-mediated transport	6	13.3%
Down- regulated (41)			
	RNA metabolic process	16	39.0%
	organelle organization	9	22%
	transcription	8	19.5%
	ribosome biogenesis	7	17.1%
	translation	7	17.1%
	transport	6	14.6%
	biological process unknown	6	14.6%

Gene hits represent the number of genes for the up- or down regulated genes belonging to the particular GO term. This value is also given as a percentage (cluster frequency) of the total number of significantly changed genes for the respective group. P-values are provided as a score of significance (cut off ≤ P 0.01).

To identify the transcription factors around which the most significant changes occur, the reporter features algorithm (Oliveira et al., 2008), using the interaction based on protein-protein interaction networks was applied (Table 8-6, hypergeometric test at P<0.01). In total, the analysis revealed 5 TFs with high degree of transcriptional regulation.

Table 8-6. Transcription factors for the maltose supplemented fermentations, based on the t-test comparison between the control and Flavourzyme supplemented fermentations.

Transcription factors	Number of neighbours	Z-score	P-value
FKS1	24	2,7	0,003
POS5	141	2,48	0,007
HAP1	24	2,47	0,008
MOT3	19	2,34	0,01
UPC2	17	2,08	0,02

The number of neighbours indicates the number of genes regulated by the particular transcription factor. Z-score represents the score of each TF, calculated based on the score of its neighbours. The P-value gives measure of significance and all results ≤0.05 are reported.

Among those, FKS1 is involved in cell wall synthesis and maintenance, POS5 is mitochondrial NADH kinase and phosphorylates NADH and NAD(+) with lower specificity and it is required for the response to oxidative stress, HAP1 is a zinc finger transcription factor involved in the complex regulation of gene expression in response to levels of heme and oxygen, MOT3 is nuclear transcription factor involved in the repression of a subset of hypoxic genes by Rox1p, repression of several DAN/TIR genes during aerobic growth, and repression of ergosterol biosynthetic genes. While HAP1 and MOT3 are involved in RNA metabolic processes and transcription, POS5 is involved in response to stress and cofactor metabolic process, HAP1 in cellular respiration, FKS1 in cell wall and membrane organization and transport and UPC2 in carbohydrate metabolic process. To account for the directionality of the reported regulations, Reporter TFs for only the upregulated or only the downregulated genes were performed. Among the five significantly altered TFs, only

MOT3 was involved in the regulation of the downregulated genes, while the rest of the TFs were involved in the regulation of the upregulated genes.

Discussion

In the food industry, enzymes can be used as alternatives for traditional chemical-based technology and can substitute the use of synthetic chemicals in many different process applications. Their advantages are associated with more specific modes of action, reduced formation of byproducts and as a result, improved environmental performance of the production process such as lower energy consumption and biodegradability of waste products.

In this study, enzyme hydrolysis experiments confirmed that the addition of the multicomponent enzyme Flavourzyme led to an increase in the available FAN, which in turn resulted in improved fermentation performance with a higher specific growth rate and more a favored flavor profile of the final beer (Chapter 7).

In addition to the importance of the assimilable nitrogen availability, the detailed metabolome and transcriptome analysis in the present study confirmed the importance of the wort sugar composition on the utilization of the supplied nitrogen. Both transcriptome and metabolome analysis revealed significantly lower impact on the Flavourzyme addition for the glucose syrup supplemented fermentations compared to the maltose ones.

Thus, the difference in the amino acids and organic acid profile among the studied fermentations were driven by the combined effect of two major events. On one side, the intracellular metabolome profile revealed higher intracellular concentration for most of the amino acids in the maltose syrup supplemented fermentations compared to the glucose ones. An increase in the range of 1 to 2 fold for the amino acids in the early exponential phase for the maltose syrup supplemented fermentations revealed that the addition of Flavourzyme influenced the amount of nitrogenous compounds in the maltose syrup supplemented wort. During the stationary phase, comparison across the metabolome profile between the glucose and maltose syrup supplemented fermentations without the addition of proteases also showed higher concentration for most of the amino acids and some of the organic

acids for the stationary phase samples of the maltose syrup supplemented fermentations. Furthermore, this difference was further enhanced with the addition of Flavourzyme.

Wort´s amino acids also have been previously classified in three groups based on the impact of the contribution of their corresponding α-keto acid analogues to the development of a balanced spectrum of their flavour compounds (Jones and Pierce, 1970). According to this classification, aspartate, asparagine, glutamate, glutamine, threonine, serine, methionine and proline belong to class 1, isoleucine, valine, phenylalanine, glycine, alanine and tyrosine belong to class 2 and lysine, histidine, arginine and leucine belong to class 3. While the concentration of amino acids from class 1 in the wort is considered to be relatively unimportant, as they can be either assimilated from the wort or synthesized *de novo*, the initial concentration of the compounds from class 2 is crucial since in the later stages of the fermentation, the synthesis of these compounds from sugars is repressed. The amino acids from class 3 are also of importance since they are delivered exclusively from wort. Thus, deficiencies in the amino acids from group 2 and group 3 will restrict the synthesis of compounds derived from the α-keto analogue of these amino acids and metabolism of their related by-products and thus affect the beer quality (Briggs et al., 2004). Addition of Flavourzyme leads to an increase in the amino acid content for mostly amino acids from group 2 and group 1, as well as histidine belonging to group 3. Thus, addition of Flavourzyme might contribute to a more balanced flavor profile of the resulting beer product.

In particular, the addition of Flavourzyme to the maltose syrup supplemented fermentations both from the early exponential and from the stationary phase, revealed an intracellular increase in the amino acids such as alanine, valine and leucine which are involved in the pyruvate and phosphoenol pyruvate metabolism. Comparison across the glucose and maltose syrup fermentations with and without Flavourzyme addition also revealed higher concentrations of these amino acids for the maltose syrup supplemented fermentations. Increase in the pyruvic acid itself, as well as most of the other TCA cycle intermediates such as fumaric, malic, succinic acid and citric acid from the early exponential phase of the maltose syrup supplemented fermentations was also observed. The increase in the concentrations of those compounds is possibly an indicator of the higher metabolic activity in the maltose syrup supplemented fermentations compared to the glucose ones (Devantier, 2005). Addition of Flavourzyme to the glucose syrup supplemented fermentations result in an increase in the intracellular concentrations of TCA cycle intermediates such as 2-oxoglutaric acid, citric acid and isocitric acid in the stationary phase. The observed TCA intermediates accumulation

inside the cell is possibly a result of amino acid catabolism. Another reason could be that high glucose concentrations lead to repressed yeast growth due to increased osmotic pressure and result in incomplete TCA cycle accumulation (Briggs et al. 2004).

Transcriptome analyses also confirmed higher impact of Flavourzyme supplementation on the maltose syrup supplemented fermentations compared to the glucose ones. While statistical analyses revealed only 6 significantly changed genes upon the addition of Flavourzyme for the glucose syrup supplemented fermentations, 86 genes were significantly changed for the maltose syrup supplemented fermentations.

Addition of Flavourzyme did not result in the same transcriptional response in the glucose syrup supplemented fermentations compared to the maltose syrup supplemented ones. Presence of high concentrations of glucose in the growth medium represses the transcription of multiple genes involved in the alternative carbohydrate and mitochondrial metabolism. This phenomenon is known as carbon catabolite repression (CCR). CCR encounters coordinated downregulation of the transcription of large groups of genes involved in metabolism of non-glucose carbon sources, a number of hexose transporters and respiration (Gancedo, 1998; Gash et al., 2002). A major role in the global regulation of CCR is played by the two nutrient signaling transducers- SNF1 and GCN2. TOR1 is another important nutritional transducer which has been implicated in the up-regulation of the general amino acid permease with broad specificity- GAP1, in the down regulation of the tryptophan and tyrosine permease- TAT2 and the high affinity histidine permease- HIP1. Recent study of Peter et al., 2006 found that transport of neutral, cationic and anionic amino acids is regulated by CCR at the protein expression and functional levels. By deletion of various genes involved in the amino acids sensing and uptake, Peter et al., 2006 proved that signaling of the activation of neutral and cationic amino acid permeases due to CCR activation is via TOR1 pathway and not through the SNF1/MIG1, GCN2 or RAS kinase pathways. The authors observed an increase in the amino acid transporter activity for all three classes of amino acids when *S. cerevisiae* was grown on alternative carbon sources as, for example, galactose media, compared to those grown on glucose media. This observation may explain the lower amino acid uptake for the glucose syrup supplemented fermentations with Flavourzyme addition, where only a small increase in the relative concentration for some of the studied intracellular amino acids (in the metabolome profile) and a low number of significantly changed genes (in the transcriptome profile) were observed compared to maltose syrup supplemented fermentations with Flavourzyme addition.

Therefore, it is possible that the observed carbon catabolite repression in glucose rich media also represses the uptake of nitrogenous compounds in the wort. As a result of such repression, the low number of significantly changed genes in the glucose syrup supplemented fermentations is a possible indication that the regulatory control of Flavourzyme addition is mainly achieved at the levels of enzyme kinetics (metabolome) and not at hierarchical (transcriptome) levels (Cakir et al., 2006).

On the contrary, for the maltose syrup supplemented fermentations, addition of Flavourzyme might have also resulted in significant changes at the levels of transcription/translation and post-translational modifications.

In conclusion, changes in the amino acid uptake when glucose or alternatives to glucose carbon sources are used, play an important role in protein synthesis and other processes of cell metabolism. For example, amino acids form intermediates for the major catabolic pathways such as acetyl CoA, pyruvate and 2-oxoglutarate (Peter et al., 2006). Thus, the wort amino acid content and utilization in combination with the wort sugar composition used is of great importance, since they will have large impact not only on the brewer´s yeast metabolism, but will also contribute to the flavour profile of the final beer. This study is also an illustration of how the combination of transcriptome and metabolome analyses can be used for "system-wide" analysis in industrial yeast fermentations for better understanding of the complex secondary metabolism and the types of regulation (hierarchical and/or metabolic) as a result of given environmental change.

References

Affymetrix: Affymetrix GeneChip Expression Analysis Technical Manual. Affymetrix, Santa Clara, CA. I*n.*; 2000.

Benjamini Y, Hochberg Y (1995) Controlling the False Discovery Rate: a practical and powerful approach to multiple testing. J R Statist Soc B, 57(1):289-300.

Bolstad BM, Irizarry RA, Astrand M, Speed TP (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics, 19(2):185-193.

Boulton C, Quain D (2006a) High gravity brewing In: Brewing Yeast and Fermentation, 2nd edn. Blackwell Science Ltd., Oxford, pp 60-63.

Briggs D, Boulton C, Brookes P, Stevens R (2004) Fermentation technologies. In: Brewing Science and Practice, Woodhead Publishing Limited, Cambridge, pp. 401-468.

Cakir T., Patil K. R., Önsan Z. Í., Ülgen K.Ö., Kildar B., Nielsen J. (2006) Integration of metabolome data with metabolic networks reveals reporter reactions. Mol. Syst. Biol. Doi:10.1038/msb4100085.

Devantier R (2005) Investigation of the mechanism behind the protease effect In: *Saccharomyces cerevisiae* in very high gravity ethanol fermentations using simultaneous saccharification and fermentation, PhD thesis, Technical University of Denmark, pp. 35-51.

Gancedo, J. M. (1998) Yeast carbon catabolite repression. Mol. Biol. Rev. 62:334-361.

Gash A.P. and Werner-Washburne M. (2002) The genomics of yeast responses to environmental stress and starvation. Func. Integr. Genomics 2:181-192.

Gautier L, Cope L, Bolstad BM, Irizarry RA (2004) Affy--analysis of Affymetrix GeneChip data at the probe level. Bioinformatics, 20(3):307-315.

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J et al. (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome Biol, 5(10): R80.

Gibson B.R., Lawrence S. J., Leclaire J. P. R., Powell C. D., Smart K. A. (2007) Yeast responses to stresses associated with industrial brewery handling. FEMS Microbiol. Rev. 31: 535-569.

Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP (2003) Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res, 31(4): e15.

Novozymes A/S, web information. Available at http://www.novozymes.com/en/MainStructure/ProductsAndSolutions/Brewing/Brewing.htm, accessed April 1, 2008.

O´Connor-Cox E, Paik J, Ingledew W (1991) Improved ethanol yields through supplementation with excess assimilable nitrogen. J. Ind. Microbiol. 8:45-52.

Oliveira A.P., Patil K.R. and Nielsen J. (2008) Architecture of transcriptional regulatory circuit is knitted over the topology of bio-molecular interaction networks. BMC Syst. Biol. 2: 17-.

Otero J. M., Panagiotou G. and Olsson L., Adv. Biochem. Engin/ Biotechnol, 108, 2007, pp. 1-40, DOI 10.1007/10_2007_071.

Panagiotou G, Kouskoumvekaki I, Jónsdóttir S. O, Olsson L (2007) Monitoring novel metabolic pathways using metabolomics and machine learning: induction of phosphoketolase pathway in *Aspergillus nidulans* cultivations. Metabolomics 3:503-516.

Pavlidis P (2003) Using ANOVA for gene selection from microarray studies of the nervous system. Methods, 31(4):282-289.

Peter G. J., Dűring L., Ahmed A (2006) Carbon catabolite repression regulates amino acid permeases in *Saccharomyces cerevisiae* via the TOR signaling pathway. J. Biol. Chem. 281:5546-5552.

Pierce JS, Jones, M (1970) Proc. 25th Cong Eur. Brew. Conv., Interlaken, p. 151.

Villas-Bôas S.G (2005) Mass spectrometry in metabolome analysis. Mass Spectr. Rev.24: 613 – 646.

Villas-Bôas S.G., Moxley J., Akesson M., Stephanopoulos G., Nielsen J. (2005) High-throughput metabolic state analysis: the missing link in integrated functional genomics of yeasts. Biochem J. 388:669-677.

9

Conclusions and future perspectives

In this study, I designed a model fermentation system and used it to systematically study the physiology (in terms of detailed growth characteristics, including intracellular trehalose content, extracellular amino acids and flavor profiles) and "-omically" (global transcriptome and metabolome analyses) characterise the most popular industrial lager beer yeast strain- Weihenstephan 34/70 at average and high gravity. In addition, its physiological behaviour and metabolome profiling was compared to two other industrial strains: the well ethanol tolerant strain AJL 3126 and the less ethanol tolerant strain AJL 2252.

This study contributed towards further elucidation of the various growth limitations imposed on the brewer´s yeast under high gravity brewing and its responses to the stressful conditions prevailing in that type of brewing. One of the challenges in high gravity brewing is that the addition of sugar syrups to increase the gravity dilutes the available nitrogen content. To approach this problem, the effect of addition of various nitrogen sources on the metabolism of strain Weihenstephan 34/70 was investigated and the beneficial effect of protease supplementation was characterised using transcriptome and metabolome analysis.

The main contributions of the work can be summarised in the following points:

i. *Detailed physiological and "omics" characterisation mapped the response to high gravity conditions*

The comparative physiological characterisation of strain Weihenstephan 34/70 at average gravity (14 °P) and high gravity (21 and 24 °P), achieved with the addition of glucose (Gl) or maltose (M) rich syrups to the 14 °P wort, underlined the effects of the various stress factors on the brewer´s yeast metabolism. It was shown that in order to minimize the effects of the negative fermentation performance of the brewer´s yeast due to increased stress at high gravity, the choice of the type of

sugar syrups used to increase the gravity is of important consideration. The use of maltose syrup to increase the gravity, assuming that there is no additional FAN supplementation, resulted in a more balanced fermentation performance in the 21 °P fermentations. However, further increase in the gravity to 24 °P lead to finding that the type of sugar syrup supplementation did not influence the growth characteristics significantly. This observation was further confirmed by intra- and extracellular metabolome analyses of the studied fermentations where many of the studied metabolites, mainly TCA cycle intermediates and their derivatives in the stationary phase were present in significantly higher concentrations for the 24 °Plato fermentations, compared to the lower gravity fermentations. Possibly, this can be explained by the combined effects of observed stuck fermentation at this gravity, resulting in restricted growth and by the eventual effect of ethanol toxicity, due to the high ethanol concentrations at the end of the fermentations.

The ethanol tolerant strain AJL3126 and the less ethanol tolerant strain AJL2252 were also physiologically characterized at average gravity- 14 °P and at high gravity- 21 °P. In general, the two strains followed similar fermentation profile compared to strain Weihenstephan 34/70 at the respective wort type and studied gravity. The exception was the poorer fermentation performance of the less ethanol tolerant AJL2252 strain, compared to the fermentation performance (higher amount of residual fermentable sugars and lower ethanol yield) of the other two strains, in 21 °P glucose syrup supplemented fermentations. In addition, intra- and extracellular metabolome analysis from the stationary phase samples and flavor compounds of the final beer also showed distinct separation for these fermentations from the rest of the studied conditions. Most of the intracellular and extracellular amino acids and TCA cycle intermediates were present in higher concentrations, also indicative of restricted growth and leakage of cellular content.

Investigation of the genome stability of lager brewer´s yeasts in terms of gross chromosomal rearrangements is very important for ensuring consistent phenotype in subsequent fermentations and is an often discussed problem in the brewing industry as their frequency, the mechanisms behind it and the consequences of it are still not well known. For this purpose, the lager beer strain Weihenstephan 34/70, was streaked out from a single stock and its karyotypes were followed during ten consecutive re-plating cycles. In this study it was confirmed that the brewer´s yeast genome does undergo gross chromosomal rearrangements. Considering the larger genome size of the brewer´s yeast and the hypothesis that some form of dosage compensation effect might take place, it

is possible that loss in chromosomal band does not affect the brewer´s yeast's phenotype, its subsequent fermentation performance and the respective quality of the beer product.

As the lager beer fermentation process is highly complex, both in terms of brewer´s yeast genome and fermentation media (wort), it is of interest to use available "-omics" techniques in order to characterise the brewer´s yeast fermentation process and relate the genotype to the phenotype.

In order to further investigate the effect of high gravity brewing on the metabolism of the lager brewer´s yeast Weihenstephan 34/70, genome wide transcription analyses were performed. With increase in gravity, down regulation of the genes involved in transcription, translation and up-regulation of the genes involved in organelle organization, RNA metabolic process and ribosomal biogenesis were observed. When accounting both for the effect of type of sugar syrup used and gravity of the wort, a number of significantly changed genes were involved in a variety of stresses such as heat shock, DNA repair, oxidative stress, trehalose biosynthesis, osmotic stress, salt stress and autophagy. Of those, the largest group of genes was involved in oxidative stress. Intra- and extracellular metabolome analysis, in addition to the physiological characterisation and characterisation of the amino acids profile and global transcriptome analysis from those fermentations confirmed that the limitation in nitrogenous compounds, in particular amino acids were mostly pronounced at 24 °Plato fermentations, resulting in incomplete fermentations characterised with lower growth rate, high amounts of residual free amino nitrogen and fermentable sugars at the end of the fermentations and accumulation to higher concentration of intra- and extracellular metabolites at the stationary phase of the fermentations.

ii. The addition of organic and inorganic nitrogen sources in high gravity beer fermentations promotes the cell growth and enhances brewer´s yeast metabolism

Nitrogen supplementation to high gravity beer fermentation is beneficial and improves the fermentation performance of the brewer´s yeast. In all cases, the fermentations with nitrogen source supplementation had a longer exponential growth phase compared to the control fermentations. Among the studied nitrogen sources, I found that the fermentations with the

multicomponent protease supplementation- Flavourzyme resulted in the highest specific growth rate, highest specific ethanol and glycerol productivities and lowest amount of residual sugars. The beneficial effect of Flavourzyme on the brewer´s yeast fermentation performance resulted also in increased initial FAN value and higher FAN uptake and improved flavour profile. The choice of nitrogenous compounds as a supplement in beer fermentation was of great importance.

The type and concentration of the supplemented nitrogen sources also influenced the flavour profile of the final beer. In this study, I observed that the most significant differences of the flavour and aroma compounds for the nitrogen supplemented fermentations were lower concentrations of acetaldehyde and slightly lower concentrations of ethyl acetate compared to the control fermentations.

iii. Use of "x-omics" characterisation reveal the beneficial effect behind the addition of proteases

The applied metabolome and transcriptome analysis were used to elucidate the effect on the addition of the multicomponent protease enzyme Flavourzyme and mapped the effect of the wort sugar composition on the nitrogen uptake. Both transcriptome and metabolome analysis revealed significantly higher impact of protease addition for the maltose syrup supplemented fermentations, while addition of glucose syrup to increase the gravity of the wort enhanced the effect of glucose repression and further inhibited the effect of the protease addition.

The unusually high amount of residual FAN at the end of the 24 °P fermentations revealed that further investigation of the other growth limiting factors when sugar syrups are used to achieve higher gravities is necessary to unravel the mechanisms behind the effects of stuck fermentations and the complexity of the metabolic yeast response to both nitrogen and other growth factor limitations. In the brewing practice, the use of glucose rich syrup as adjunct is substancially cheaper compared to the use of maltose rich syrup. In addition, considering the need for constant optimization of the brewing fermentation process and further economic savings, it is of great industrial relevance that the effect of glucose repression in beer fermentation is investigated in

details and optimal sugar compostion in terms of mono-/di-/tri and polysscharides in addition to optimal presence of other nutrients is achieved such that both the quality of the beer product is not compromised, but maximum economic savings in terms of substrate price are achieved.

The question whether or not gross chromosomal rearrangements will influence phenotypically the fermentation performance of brewer´s yeast is still open. As the brewing community is expecting the completion of and publicly available sequence for strain Weihenstephan 34/70 and other popularly used lager beer yeast strains, it is of great interest that chromosomal translocations continue to be investigated in the future and karyotypes with observed gross chromosomal rearrangements undergo further detailed phenotypical characterisation in subsequent fermentations.

This thesis shows that system biology tools such as transcriptome and metabolome analysis can be used to better characterize industrial fermentation processes, despite the often complicated and dynamic nature of the industrial strains' genomes and the high complexity of the industrial media used. In this respect, the present study also serves as an example that "omics" techniques can be more broadly used in the future to characterize industrial products and processes for different organisms.

The additions of proteases have also showed that they can be successfully used in high gravity fermentations to increase the available nitrogen content and thus improve the brewer´s yeast fermentation performance. As in the modern brewing world there is constant need for further process optimization and demand for new products, the use of enzymes in brewing is becoming increasingly popular. This work is of great relevance for the brewing industry and gives an example to be followed where application of many new enzymes in the beer fermentation process can be investigated.